CAUSES AND CONTROL OF LOW F/M BULKING IN LONG SLUDGE AGE NUTRIENT REMOVAL ACTIVATED SLUDGE SYSTEMS

by

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For my Parents

for their lifelong unselfish sacrifices

ABSTRACT

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A problem plaguing the greater proportion of nutrient removal activated sludge systems treating municipal sewage, is the prolific growth of filamentous organisms (bulking), which inhibits the rate of sludge settling in the clarifier (secondary settling) stage of treatment and reduces plant throughput.

To find the causes of filament proliferation, a four–stage investigation was conducted: (1) A review of literature indicated that the promoted method for control of bulking in carbonaceous removal systems (the selector reactor) does not control bulking in nutrient removal systems. (2) From a laboratory–scale experimental programme it was concluded that low F/M filaments proliferate when sludge is subjected to alternating anoxic–aerobic conditions with nitrite present. (3) A review of the biochemistry of respiration led to the formulation of a biochemical model for facultative organism respiration, an important aspect of which is a series of mechanisms that give rise to inhibition of aerobic respiration following anoxic conditions. Inhibition of respiration was measured in activated sludge with aerobic batch test procedures. (4) Application of the biochemical model to filaments and floc–formers allowed the formulation of a biochemical/microbiological (bulking) model to describe the prolific growth of filamentous organisms. The model was tested and verified at laboratory–scale.

From the biochemical and bulking models, configuration modifications and operational control procedures were identified and tested, by means of which bulking can be avoided at the design stage or controlled in existing systems. The procedures produced positive results.

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SYNOPSIS

Virtually since the inception of the activated sludge system for wastewater treatment, the presence of filamentous organisms in the mixed liquor has constituted a problem in liquid–organic solids separation in the clarifier (secondary settling tank) stage of the process. High proportions of filaments cause interaction between sludge floc material, reducing the rate of sludge settleability. Increasing sludge settleability would allow an increase in treatment capacity in plants in which sludge bulking restricts throughput and would allow a reduction in secondary settling tank size in future plant designs. These benefits are the motivation for the research conducted in this investigation.

In the 1920s and 1930s bulking did not appear to be widespread and was confined, principally, to plants treating effluents derived from some industries. After the Second World War, from about 1950, bulking became a widespread problem. Causes for this have been variously attributed to (i) greater proportions of industrial waste in the influents, (ii) higher standards for the treated effluents requiring lower organic loading rates per unit of mixed liquor (longer sludge ages, lower F/M ratios), and (iii) changes in plant configuration and mixing mode (in particular, deviation from the plug–flow regime to completely mixed reactors, single or in–series).

Before the Second World War, the approach to solving the bulking problem was to look for factors such as sewage characteristics, and plant configuration and operation that appeared to be associated with filament proliferation and to control bulking by removing the cause. A distinctive feature of the era was that bulking was attributed universally to proliferation of the filament *Sphaerotilus natans*. After the Second World War there was increasing recognition that a variety of filamentous organisms could proliferate in the sludge and give rise to bulking. About 30 filamentous organisms were recognised, of which about 11 were widely present and thus of technical importance. Many of these filaments were previously unknown and hence not included in identification keys. Practical procedures for identification of these organisms in mixed cultures were developed principally by Eikelboom and his

conducted. The hypothesis was widely investigated by many researchers with positive outcomes.

In South Africa, nitrification–denitrification (ND) and nitrification–denitrification biological excess phosphorus removal (NDBEPR) in activated sludge systems requires long sludge ages (15 – 25 days) and additionally the incorporation into aerobic systems of anoxic zones (for ND systems) and anoxic and anaerobic zones (for NDBEPR systems). These plants show a great propensity to bulk, with typical filaments being type 0092 and *Microthrix parvicella*, filaments that were not common in European and North American aerobic plants. In terms of Jenkins categorization these filaments develop in low F/M systems. With the increase in number of ND and NDBEPR systems in Europe and North America, development of bulking sludges with filaments of the low F/M category has become an increasing problem.

The success of Chudoba's selection hypothesis and the paucity of information on its effect in low F/M systems, prompted enquiry into the selector effect in long sludge age aerobic and anoxic–aerobic systems by the Water Research Group of the University of Cape Town. The outcome of this enquiry can be summarized as follows:

In long sludge age systems,

- the selector effect appears to have no significant effect on the proliferation or suppression of low F/M filaments in aerobic or alternating anoxic–aerobic systems,

- low F/M filaments will not proliferate in completely aerobic systems,

- a *necessary* condition for low F/M filament proliferation is alternation between anoxic and aerobic states, however,

- alternation between anoxic and aerobic states is not a *sufficient* condition for bulking because some anoxic–aerobic systems bulk with low F/M filaments whereas others do not.

These findings constituted the state of knowledge on bulking in long sludge age

with low DSVI values developed.

- Systems developed their highest DSVIs when the aerated mass fraction was between 30 and 40%.

- Systems fed substrate with high TKN/COD ratios (resulting from ammonium addition to the influent) invariably developed sludges with high DSVI values.

- Systems fed substrate with low TKN/COD ratios developed sludges with low DSVI values.

- Nitrate and nitrite addition to the anoxic zone of alternating anoxic–aerobic systems led to an increase in DSVI and with their removal, a decrease in DSVI.

- Increases and decreases in DSVI were accompanied by decreases and increases respectively in VSS.

- Increases in DSVI were accompanied by decreases in COD and nitrogen mass balances and decreases in DSVI were accompanied by increases in COD and nitrogen mass balances.

- Sludge age (F/M ratio) appeared not to influence sludge settleability in alternating anoxic–aerobic systems; the DSVI remained high at sludge ages between 5 and 20 days.

- The filamentous organism types which were present in sludges with high DSVIs were invariably those categorized as low F/M, which dominate in full–scale systems.

- In systems with sludges exposed to alternating anoxic–aerobic conditions which developed a high DSVI, the frequency of alternation (from cycle times of 20 minutes to 3 days) had little or no effect – the DSVI remained high.

From the experimental programme, it was concluded that:
Low F/M filaments proliferate to their greatest extent with exposure of sludge to anoxic–aerobic alternation in which the aerated mass fraction is between 30 and 40 percent of the total and nitrate and/or nitrite is present throughout the unaerated

diversion of electrons from the anoxic respiration pathway to the aerobic respiration pathway.

(3) Under anoxic conditions (with nitrate/nitrite present) and with slowly biodegradable substrate also present, one of the intermediates produced in the denitrification pathway (i.e. nitric oxide – NO) accumulates, and in switching from anoxic conditions to aerobic conditions, nitric oxide interacts with the aerobic electron transferring complexes the cytochrome oxidases, inhibiting electron transfer to oxygen, thereby inhibiting substrate utilization (determined by measuring oxygen utilization rate – OUR) under aerobic conditions.

(4) Inhibition of OUR under aerobic conditions results in denitrification of nitrite but not nitrate under aerobic conditions.

(5) With an adequate supply of electrons from readily biodegradable substrate, under anoxic conditions, nitric oxide is reduced as rapidly as it is formed; it does not accumulate, and inhibition is not induced under subsequent aerobic conditions.

(6) Under continuous aerobic conditions, the aerobic enzymes (cytochromes *o* and aa_3) are synthesized, and denitrifying enzymes (the reductases) are not synthesized and degrade with time.

(7) Under continuous anoxic conditions the reductases, and the oxidase cytochrome *o* is synthesized, but the principal aerobic enzyme, cytochrome aa_3 is not synthesized and degrades with time.

The biochemical model was examined experimentally.

To examine inhibition of substrate utilization – see (3) above – an aerobic batch test procedure was developed. Sludge from a two–reactor nitrification–denitrification (2RND) system was subjected to anoxic conditions with nitrite present to induce inhibition through accumulation of intracellular nitric oxide. Under aerobic batch test conditions, inhibition of OUR was proportional to the concentration of nitrite present.

substrate utilization rates and filament growth is restricted. In nitrification–denitrification (ND) and nitrification–denitrification biological excess phosphorus removal (NDBEPR) activated sludge systems, competition between filaments and floc–formers for mutually growth–limiting substrate is influenced by inhibition of the floc–formers substrate utilization under aerobic conditions. Under anoxic conditions, in utilization of substrate, the floc–formers execute the denitrification of nitrate through each of the denitrification intermediates to dinitrogen as follows:

$$NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$$

nitrate → nitrite → nitric oxide → nitrous oxide → dinitrogen

In the absence of, or at low concentrations of readily biodegradable substrate, the intermediate, nitric oxide is accumulated intracellularly in the floc–formers. In the subsequent aerobic zone, intracellular nitric oxide inhibits the utilization of oxygen by floc–forming organisms as a result of the interaction of nitric oxide with the enzymes specific to aerobic respiration, the cytochrome oxidases, cytochrome o and cytochrome aa_3. Under these conditions, floc–forming organisms are inhibited in aerobic respiration. The inhibition of aerobic respiration causes electrons to be redirected to the nitrite–, nitric oxide– and nitrous oxide reductases for the reduction of the nitrogen oxides specific to these reductases (aerobic denitrification); this mechanism continues as long as nitrite remains available. Thus, under aerobic conditions a low concentration of nitrite maintains the intracellular accumulation of nitric oxide (and thereby the inhibitory effect) and higher concentrations of nitrite exacerbate the inhibitory effect. The inhibition of aerobic respiration and the phenomenon of aerobic denitrification in floc–formers results in lower substrate utilization rates and lower nett energy yields.

In contrast to floc–formers, the filamentous organisms are nitrate reducers and execute only part of the denitrification pathway, i.e. the reduction of nitrate to nitrite as follows:

$$NO_3^- \rightarrow NO_2^-$$

They do not accumulate nitric oxide and therefore are not inhibited in aerobic respiration in the subsequent aerobic zone.

by manipulation of the a– (aerobic–anoxic) recycle to ensure that the nitrate load to the anoxic zone immediately prior to the aerobic zone (i.e. the 2nd anoxic zone in MUCT systems or the primary anoxic zone in modified Bardenpho, UCT and Johannesburg systems) never exceeds the denitrification potential of that zone.

Summary

In closure, this investigation has contributed significantly to knowledge regarding the problem of low F/M filamentous organism bulking. When the investigation commenced, the conventional practice for control of filaments (the selector reactor), although successful for some filament types, had been discredited as a control measure for low F/M filaments. With this as a background, the investigation has:

- Investigated the effect of numerous factors on low F/M filament proliferation through an extensive experimental programme; the specific conditions resulting in low F/M filament proliferation were elucidated and on the basis of these conditions a name change was suggested, from low F/M to AA filaments.

- Formulated a conceptual biochemical model for facultative organism respiration as a consequence of an extensive review of the literature of facultative organism respiration under a variety of conditions.

- Verified the biochemical model with activated sludge subjected to specially developed aerobic batch test procedures.

- Developed a conceptual biochemical/microbiological (bulking) model to explain the proliferation or not of AA filamentous organisms under virtually all conditions encountered in activated sludge systems.

- Verified the bulking model by controlling and inducing bulking incidents in laboratory–scale NDBEPR systems and by applying the model to all significant laboratory–scale experimental results produced during the exploratory experimental investigation.

- Proposed, and tested at laboratory–scale, procedures for control of bulking at the design stage and during operation of full–scale ND and NDBEPR systems.

It is concluded that these developments have established a basic framework which

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LIST OF SYMBOLS

SYMBOL	DESCRIPTION
AA	Anoxic–aerobic
ADP	Adenosine diphosphate
AMO	Ammonium monooxygenase
ATP	Adenosine triphosphate
ATPase	Adenosine triphosphatase
aq	Aqueous
BEPR	Biological excess phosphorus removal
COD	Chemical oxygen demand (mg/ℓ)
Cyt	Cytochrome
Cu	Copper
BT	Batch test
CoA	Coenzyme A
d	Days
h	Hours
DBT	Denitrification batch test
DENOX	Denitrification of nitrogen oxides
DO	Dissolved oxygen (mgO/ℓ)
DPNH	Diphosphopyridine nucleotide (reduced)
DSBT	Defined substrate batch test
DSVI	Dilute sludge volume index (mℓ/g)
E_h	Redox potential (mV)
ETP	Electron transport pathway
F	Faradays constant (cal/volt)
$\Delta\mu_H$	Change in proton motive force
$\Delta\psi$	Change in electrical charge (volt)
FAD	Flavin adenine dinucleotide (oxidized)
$FADH_2$	Flavin adenine dinucleotide (reduced)
Fe	Iron
FeS	Iron–sulphur
F/M	Food/microorganism (mgCOD/gVSS)
F_p	Flavoprotein

ox	Oxidized
p	Phosphorus
polyP	Polyphosphate
PWWF	Peak wet weather flow
Q	Ubiquinone (oxidized)
QH_2	Ubiquinol (reduced)
R	Universal gas constant
RBCOD	Readily biodegradable COD (mg/ℓ)
red	Reduced
R_s	Sludge age (d)
S	Sulphur
s	Seconds
SBCOD	Slowly biodegradable COD
$SSVI_{3,5}$	Stirred settling volume index (g/ℓ)
SVI	Sludge volume index
T	Temperature
TCA	Tricarboxylic acid
TKN	Total Kjeldahl nitrogen concentration (mgN/ℓ)
TMPD	N,N,N'–N'–tetramethyl–ρ–phenylenediamine
UCT	University of Cape Town
μm	Micrometers
μ_{max}	Maximum specific growth rate
VSS	Volatile suspended solids (mg/ℓ)
2RND	Two–reactor nitrification–denitrification
°C	Temperature in degrees Celcius

CHAPTER 1

INTRODUCTION

In the Republic of South Africa, the need for high quality effluents from sewage treatment plants is two–fold. Firstly, the country is rapidly approaching the point of maximum economic exploitation of conventional water resources, and high quality treated effluents are increasingly becoming a resource in their own right to augment conventional resources. Secondly, the country faces a deterioration in quality of the existing conventional water resources from the discharge of inadequately treated wastewaters to rivers, lakes and dams. The quality of effluents from wastewater treatment plants has, in consequence, become a matter of national importance.

The quality demanded of effluents, chemically and bacteriologically, has evolved within the constraints of the treatment technologies available and with due regard for the requirements of the major water users in the country, i.e. the domestic, agricultural, industrial, recreational and conservational sectors. As a consequence of limited water resources, virtually every river and dam is utilized to some degree so that the maintenance of a "healthy" state in all the rivers and storage dams is a matter of vital importance.

The evolution of South African Water Law [1]

The quality of effluents in wastewater treatment is controlled by the principles of South African water law. South African water law has evolved through a fusion of Roman–Dutch law from Dutch settlement of the Cape, and English law from later British rule. Under Dutch rule, the Roman–Dutch principle of *primus fluminus* applied, which vested the State with extensive control over the use of surface waters. Under British rule, the English legal principle of riparian ("on or of the river bank") ownership vested the State with minor control.

With time, unconditional riparian ownership of water came to be inadequate to ensure optimal use of the country's water resources and consequently there was a

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[1] For a more extensive review of this topic, see Chapter 8.2 of Management of the Water Resources of the Republic of South Africa. Department of Water Affairs, 1986. CTP Book Printers, Cape Town.

Research into eutrophication during the 1960s had brought about a realization that, for many water bodies, phosphorus rather than nitrogen is more likely to be the growth limiting nutrient. Also, research with ND plants in the 1970s had led to the development of a process modification that facilitated biological phosphorus removal in addition to nitrogen removal, namely the nitrification–denitrification biological excess phosphorus removal (NDBEPR) system. Although this technology was not well developed, in a large measure its availability led to the promulgation of the Special Standard for Phosphate (1980); however the legislation permitted a 5 year period of grace before its enforcement to allow further development and application of the technology. Although phosphorus could be removed with physical–chemical methods, the associated increase in the salt concentration of the treated effluent was considered a grave disadvantage, which limited the reuse value of the water and would exacerbate the mineralization problems already apparent in South Africa's surface water. The biological method had the distinct advantages of lower cost without the higher effluent salt concentrations associated with chemical addition. To achieve maximum P removal by biological means, a number of versions of NDBEPR systems have been developed in South Africa for wastewaters with different influent characteristics, namely the 5–Stage Bardenpho, UCT, Modified UCT, and Johannesburg systems.

<u>A problem encountered in ND and NDBEPR activated sludge systems</u>

The biology, biochemistry, kinetics and stoichiometry of the processes in the ND and NDBEPR plants are now understood to such a level that within the temperature range in South Africa (14 to 24 °C) the system can be designed with confidence to meet the required effluent standards. Indeed, in South Africa the NDBEPR system is given prime consideration whenever a new wastewater treatment plant is planned; this system has come to be looked upon as the standard biological system for medium– and large–scale wastewater treatment plants. In these plants there may be a need for chemical addition, but only in support of, or to augment nutrient removal where the biological process alone cannot achieve the required effluent quality. However, experience with ND and NDBEPR plants in South Africa (and elsewhere) has brought to light a behavioural response that has a significant effect on the economics of design of the system – the systems are prone to produce sludges that settle poorly in the clarifier (secondary settling tank) stage of treatment, where the sludge and treated effluent are separated by gravity sedimentation settling.

systems in South Africa, but the problem is not confined to these systems and country; it has been encountered worldwide in many different kinds of activated sludge system, including ones that do not have unaerated zones, i.e. completely aerobic systems. Indeed, research into filamentous bulking control outside of South Africa has been focussed principally on the problems associated with completely aerobic systems. In these investigations it has been found that a number of different filamentous organisms contribute to the bulking condition and considerable effort has been directed at identifying the conditions in the system that give rise to proliferation of the different filamentous organism types. The outcome of this research indicated that the filaments which give rise to bulking in aerobic systems are not the same as those in ND and NDBEPR plants. This, and other results led to the categorization of the filaments in terms of the condition that *apparently* gives rise to their proliferation, viz. low dissolved oxygen (DO), low food/microorganism (low F/M) ratio, septic wastewater, nutrient deficiency, and low pH. In terms of this categorization, the filamentous organisms that proliferate in ND and NDBEPR systems belong mainly to the low F/M category. This categorization seemed reasonable because the ND and NDBEPR plants invariably are low F/M (equivalently – long sludge age) plants.

As a consequence of the dominance of low F/M filaments causing the bulking problems in ND and NDBEPR plants, research was commenced to evaluate the strategy that was promoted for the control of the low F/M filaments. This strategy is based on the so–called selector effect.

A selector effect is induced in an activated sludge when a readily biodegradable COD (RBCOD) concentration gradient is established in the system by modifying the mixing regime of the system by introducing plug–flow conditions, compartmentalization, or a selector reactor, the last being a small reactor receiving the sludge return and influent flow before the main aeration reactor. With these modifications, the floc–formers in the activated sludge acquire a high RBCOD uptake rate allowing them to successfully compete for RBCOD substrate against the filamentous organisms. However, when a selector (either aerobic or anoxic) was placed ahead of an intermittently aerated reactor mimicking an Orbal or Carousel nitrification–denitrification system, the selector effect was found to be not effective for controlling the proliferation of the low F/M filaments. Furthermore, anaerobic reactors in the NDBEPR systems, which in effect serve the same function as the selector reactor in that they also allow preferential uptake of RBCOD (or rather its

was examined with regard to the electron transport pathways of facultative organisms. The pertinent information has been included in extensive reviews in Appendix C (dealing with the electron transport pathways of facultative organisms) and Appendix D (dealing with the effect of the presence and absence of oxygen on the electron transport pathways).

To complete the review of respiration of organisms in activated sludge from a biochemical standpoint, the biochemical mechanisms of nitrifying organisms are reviewed in Appendix E.

CHAPTER 2

REVIEW OF BULKING

ABSTRACT

The course of change in perception regarding the factors responsible for filamentous organism bulking in activated sludge systems is examined. The conceptual and experimental work responsible for identification of the conditions resulting in the organism classification category "low F/M", are described. The chapter concludes with a summary of work conducted by the UCT Water Research Group, including a discussion of the conditions under which selector reactors are successful or not, elucidation of an experimental artifact present in many laboratory–scale systems which is often the cause of erroneous conclusions regarding low F/M bulking, and identification of one set of conditions which is necessary for low F/M bulking to occur.

2.1 INTRODUCTION

Activated sludge is a biocenosis of microorganisms that includes an extremely large number of species. The variety of species that develop and their concentrations, are essentially governed by four factors: composition of the influent substrate, environmental conditions imposed, species metabolism, and species interaction.

Substrate consists of (1) organic material for heterotrophic organism growth and energy generation, (2) inorganic materials that serve as an energy source for autotrophic growth and, (3) nutrients which are essential for the formation of the metabolic material and the generation of energy. The organic material is present in a variety of forms: chemical – fats, proteins and carbohydrates; physical – soluble and particulate; biodegradable – readily and slowly biodegradable and unbiodegradable. The inorganic materials include the nutrients nitrogen and phosphorus and a variety of trace nutrient elements and growth factors.

The spectrum of environmental conditions are: mixing regime (plug, intermediate or completely mixed); reactor configuration (in–series or single reactors); oxygen present or absent to give anaerobic, anoxic and aerobic states; cyclic loading of substrate and nutrients; organism retention time (sludge age); temperature; pH; and

if filamentous organisms are present in a "reasonable" concentration. If absent, pin–point flocs form which may lead to inefficient solid–liquid separation with floc escaping in the effluent. However, if present in excess, the filaments bind separate flocs in a web–like structure which impedes the separation, to give rise to the phenomenon known as bulking sludge.

The bulking behaviour of a sludge has come to be defined in terms of the settling properties of the sludge, not in terms of the effluent quality from the settlers; widely used parameters are the sludge volume index (SVI), or the diluted sludge volume index (DSVI) or the stirred settling volume index at 3,5 g/ℓ ($SSVI_{3,5}$). Of these parameters, the DSVI and $SSVI_{3,5}$ give improved correlation with the settling behaviour in the secondary settling tank over that provided by the SVI (Ekama and Marais, 1986a).

Defining bulking in terms of the settling properties of the sludge is necessary in order to eliminate situations where sludge loss to the effluent is due to inadequate hydraulic design of the secondary settling tank or due to pin–point floc formation (good settling but poor flocculation) from toxic materials or very low loaded (very long sludge age) aerobic systems. With some influents (usually industrial) at relatively short sludge ages, bulking may occur due to massive development of zooglea. Consequently microscopic examination of sludge is an essential requirement, along with the settleability test, to establish the nature of the bulking incident.

In design of the secondary settling tank, the requirement to accommodate bulking sludges has significant technical and economic consequences. Sludge settleability governs the daily flow and load that can be treated in an activated sludge plant. For a particular plant the influent peak wet weather flow (PWWF) sets the overflow rate (m/h) in the secondary settling tank, and the daily mass of COD treated and sludge age determines the sludge mass in the biological reactor. Ekama and Marais (1986a) showed that a sludge with a DSVI of 150 mℓ/g can be handled satisfactorily in the settling tank up to a maximum overflow rate of 1 m/h at a mixed liquor suspended solids (MLSS) of 3,5 g/ℓ. Should the DSVI deteriorate to 200 mℓ/g the maximum overflow rate reduces to 0,6 m/h at 3,5 g/ℓ, or to maintain the same overflow rate (at 1 m/h), the reactor MLSS must be reduced to 2,4 g/ℓ – with a DSVI increase from 150 to 200 mℓ/g, about 33 percent less flow and load can be treated in the plant. In contrast, if the DSVI is reduced from 150 to 100 mℓ/g

nitrogen contents and if these are not removed they add to the phosphorus input to the dams and exacerbate the problem of eutrophication of these water bodies.

As mentioned earlier, these nitrogen (N) and phosphorus (P) removal plants have in–series configurations with anaerobic, anoxic and aerobic reactors, inter–reactor recycles and are operated at sludge ages ranging from 15 to 30 days which, from a European perspective, would be considered to be very long sludge ages. Basically, there are two types of biological N and P removal systems, nitrification–denitrification (ND) for N removal only and nitrification–denitrification biological–excess–phosphorus removal (NDBEPR) for both N and P removal, the latter also called a nutrient removal system. In a survey of 111 wastewater treatment plants in South Africa, Blackbeard *et al.* (1986) found that 62 produced bulking sludges. Of the 111 plants surveyed, 33 were nutrient removal and the balance, N removal, either intentionally or inadvertently. In a subsequent survey, Blackbeard *et al.* (1988) focused attention on nutrient removal plants. These plants have become the preferred method for removing N and P from municipal wastewaters in South Africa. Today there are about 45 nutrient removal plants in operation, with sizes ranging from 2 to 150 Mℓ/d, with a total design capacity of about 1200 Mℓ/d. From the survey on bulking and foaming in nutrient removal activated sludge plants in South Africa, Blackbeard *et al.* (1988) found that of 33 nutrient removal plants investigated, 27 produced bulking sludges.

From the review above, clearly, bulking due to filamentous organisms has a long history of occurrence and at present still constitutes a major problem in activated sludge systems. Bulking is encountered over virtually the entire spectrum of systems, from short and long sludge age aerobic systems operating in plug flow, in–series and completely mixed regimes, to long sludge age anoxic–aerobic and anaerobic–anoxic–aerobic nitrogen and phosphorus removal systems.

In earlier reports on bulking some authors appeared to view bulking by filamentous organisms as an invasive sick condition of the sludge, for example, Smit, (1934) speaks of "....the state of bulking in a diseased sludge caused by the development of threadlike organisms." Donaldson (1932) speaks of filamentous organisms as "....the weeds of activated sludge." This point of view is echoed even in recent times when at a conference a participant spoke of bulking as the AIDS of activated sludge.

environmental conditions in the activated sludge plant which would inhibit or suppress the growth of the filamentous organisms. If successful, the method would provide a permanent solution to the particular bulking situation. Accordingly, this review will focus on the more desirable specific control of bulking.

2.5 CAUSES OF BULKING

In the 1920s and 1930s bulking was widely imputed to be due to *Sphaerotilus natans*. According to Smit (1934), it was Ruchhoft and Watkins (1928) who first succeeded in "....cultivating an organism [*S.natans*] which in pure culture closely resembled the threads present in bulking sludge". Smit (1934) appears to have been the first to show that filamentous species other than *S. natans* could be present in a bulking sludge.

Efforts at identifying the causes of bulking and its possible amelioration has in most instances followed a practical route by attempting to link bulking to some factor in the influent, the design or operation of the plant. Examples are:

Carbohydrate in the influent was implicated in bulking Scott (1928), but Smit (1934) showed that this would apply only in influents high in carbohydrate and could not explain bulking with a normal sewage influent which usually has a low carbohydrate content.

Donaldson (1932) attributed the cause of bulking to the mixing régime in aeration tanks. He suspected that filamentous organism growth in the rectangular diffused aerated tanks was due to back mixing, thereby changing the intended plug-flow regime towards a completely mixed one. As a solution he proposed that the tanks should be baffled.

Tomlinson (1976), from an extensive survey of occurrence of bulking in England, on 65 plants, attempted to identify some of the causes of bulking, *inter alia*, by statistical based analysis. From questionnaires to operators of these plants he could identify no "... overall correlation between the occurrence of bulking and any of the parameters which are normally measured, or specified in design, for example sludge loading, sludge age, retention time, power input, dissolved oxygen concentration, temperature, proportion of industrial waste waters, sewage septicity." He did, however, establish that the mixing regime influenced bulking – plants employing plug flow configurations were less prone to bulking than plants employing complete

whereby reasonably reliable identification of filaments could be attained from microscopic studies, staining, etc. Jenkins *et al.* (1984a) produced a manual that built on the work of Eikelboom and van Buijsen. This manual describes in detail the quick practical procedures for identifying filaments and also gives advice on their occurrence and suggestions for their amelioration. This manual has proved of great value in bulking research in South Africa.

About 30 filamentous species have been reported to contribute to bulking in activated sludge systems. Of these, 11 are of importance. Their dominance in activated sludge mixed liquors is dependent in varying degrees on the environmental conditions imposed on the plant by its design and operation, and the influent sewage composition. Bulking surveys in different countries have indicated the sensitivity of the organism population to differences in plant design, plant operation and influent characteristics. Based on 226 samples from 78 plants in the United States, Strom and Jenkins (1984) list the filaments forming the major component in sludges that bulk in decreasing order of occurrence: type 1701, *Nocardia* species, *Haliscomenobacter hydrossis*, types 0041, 021N and 0092, *S. natans*, *Microthrix parvicella*, and others. The ATV Working Group (1989) in Germany lists the following filamentous species in decreasing order of occurrence: Type 021N, *M. parvicella*, type 0041, *S. natans*, *Nocardia* species, *H. hydrossis*, *Nostocoida limicola*, type 1701, and other lesser occurring ones. Blackbeard *et al.* (1986, 1988) in South Africa lists the 6 most frequently dominant filaments in nitrogen and nutrient removal (long sludge age) plants, in decreasing order of occurrence: types 0092, 0675, 0041, *M. parvicella*, types 0914 and 1851. The lists for the United States and Germany are not dissimilar, but both differ substantially from that for South Africa. Plants in Germany and in the United States are principally aerobic with relatively high loading factors (high F/M) or equivalently short sludge ages. Plants in South Africa have sludge ages that can be up to an order of magnitude higher than some plants in the United States and in Germany (i.e. very low F/M) and always incorporate anoxic–aerobic or anaerobic–anoxic–aerobic zones. Whereas the microbiological population structures that develop in the United States and German plants could be expected to differ not too greatly, the significant differences in environmental factors imposed on the organisms in ND and NDBEPR plants in South Africa should give rise to a population structure that differs greatly from those in European and American plants.

With the objective of developing a practical procedure to identify filaments,

Table 2.1: Dominant filament types as indicators of conditions causing activated sludge bulking (from Jenkins *et al.*, 1984a).

Suggested causative conditions	Indicative filament types
Low DO	type 1701, *S. natans*, *H. hydrossis*
Low F/M	*M. parvicella*, *H. hydrossis*, *Nocardia* sp., types 021N, 0041, 0675, 0092, 0581, 0961, 0803
Septic Wastewater/Sulfide	*Thiothrix* sp., *Beggiatoa* and type 021N
Nutrient Deficiency	*Thiothrix* sp., *S.natans*, type 021N, and possibly *H. hydrossis* and types 0041 and 0675
Low pH	fungi

surrounding the organism, as originally conceived by Chudoba *et al.* (1973a,b). Hao *et al.* (1983) and Lau *et al.* (1984) confirmed the work of Palm *et al.* (1980). From dual species studies they showed that low DO filaments (*S. natans*, type 1701) and floc–formers can be selectively grown by manipulating the DO concentration – if high, the floc–former dominates, if low, the filament dominates.

With regard to bulking in long sludge age (low F/M) systems, Chudoba *et al.* (1973a,b) tested the selection criterion with pure soluble substrates: They controlled the substrate concentration surrounding the organism by having different configurations for the activated sludge system. For example, in a completely mixed single reactor system, the substrate concentration would be low throughout the reactor whereas in a multi–reactor plug–flow system, the substrate concentration would be high in the upstream section and low in the downstream section. They found that in aerobic completely mixed single reactor systems, filamentous organisms proliferated causing bulking, whereas in aerobic multi–reactor plug–flow systems filamentous organisms did not proliferate and a good settling sludge was maintained. From this work, Chudoba *et al.* (1973b) developed the selector reactor for bulking control. The selector reactor is a small aerated reactor upstream of the main aeration reactor and receives the influent and underflow recycle. In the selector reactor, the substrate concentration is high and, in terms of the selection criterion, the floc–formers should grow faster than the filaments and usually will utilize practically all of the soluble substrate. The mass of soluble substrate that passes through the selector unutilized is a very small fraction of that available to the floc–formers in the selector so that filament growth will be restricted and insufficient to cause bulking. The findings of Chudoba were supported by a number of investigators; non–bulking sludges were produced in *aerobic* systems with:

(1) Selectors ahead of the main reactor (Grau *et al.*, 1982; Lee *et al.*, 1982; Jenkins *et al.*, 1983; Ekama and Marais, 1986b; Still *et al.*, 1986; van Niekerk, 1985; van Niekerk *et al.*, 1987);

(2) compartmentalization of the aeration reactor while maintaining continuous feeding of waste water (Chudoba *et al.*, 1974; Rensink *et al.*, 1982; Wu *et al.*, 1984);

(3) batch or intermittent feeding to completely mixed aeration basins (Houtmeyers, 1978; Houtmeyers *et al.*, 1980; Verachtert *et al.*, 1980; van den

research, controlling bulking in low F/M systems was the focus, rather than controlling bulking by low F/M filaments. These are two distinctly different objectives because bulking in a low F/M system is not necessarily caused by low F/M filaments. It is necessary to distinguish clearly between the two situations: low F/M system bulking is bulking in a low F/M system with the filaments causing the bulking unspecified, i.e. could be *S. natans*; low F/M filament bulking is bulking caused specifically by the filaments grouped into the low F/M category.

Chudoba's Selector Theory – anoxic–aerobic systems

The system modification approach for bulking control in low F/M (long sludge age) systems also was applied by incorporating anoxic selectors in N removal activated sludge systems. The need for this arose from the necessity for denitrification for N removal. If an aerobic selector receiving the influent and underflow recycle streams is placed upstream of the nitrification–denitrification system, the influent RBCOD will be utilized in the aerobic selector. This will result in a significant loss in denitrification – as much as 50 percent – because the influent RBCOD will be utilized in the selector with oxygen rather than with nitrate in the main reactor. If the selector effect can be stimulated in an anoxic selector, the RBCOD will be utilized with nitrate as electron accepter and then the conditions for good N removal and selector bulking control would be met simultaneously.

In evaluating anoxic selectors *per se*, for bulking control in low F/M (long sludge age) activated sludge systems receiving real sewage (with or without acetate or glucose supplementation), Lee *et al.* (1982) reported that incorporation of two anoxic selectors in series, each 1/74th of the total system volume, *did not* control bulking. They sized the selectors in accordance with the volume that would be required to control bulking with aerobic selectors. Based on measurements of soluble COD through the system, they found that not all the soluble biodegradable COD was taken up in the selectors. The leakage of soluble biodegradable COD into the aerobic zone was suggested as the cause for the ineffectiveness of the anoxic selectors. Shao (1986) found that the uptake rate of RBCOD is slower under anoxic conditions than under aerobic conditions so that anoxic selectors should be sized larger than aerobic selectors.

Anoxic selectors normally would be installed upstream of in–series anoxic–aerobic reactors in order to obtain adequate denitrification. This raised the question of whether the amelioration of bulking was due to the selector effect or, perhaps,

plants. Furthermore, even the lowest SSVI in the laboratory units is higher than the highest SSVI in the full–scale units. It is not possible to isolate the causes for this disparate behaviour, but a suggested cause is that the laboratory–scale units were seeded with filamentous growth generated in the influent line. Such seeding in a pilot–scale study has been reported by Gabb *et al.* (1985). Unfortunately Cooper *et al.* (1977) did not report on the filamentous organisms found in the laboratory–scale study. It does, however, point to an important factor in utilizing laboratory–scale studies to seek corrective procedures to be applied at full–scale: it is imperative that the laboratory–scale study produces the same filament population as the full–scale study. If this is not achieved the laboratory–scale study loses much of its value as a tool for testing control procedures proposed for the full–scale plant.

In studies on anoxic–aerobic systems, Bailey and Thomas (1975) and Arkley and Marais (1981) found that as the anoxic mass fraction of the upstream anoxic reactor increased, the sludge settleability deteriorated. Arkley and Marais (1981) studied a two in–series reactor system in which the total volume remained constant, the first reactor anoxic and the second aerobic; both completely mixed with a system sludge age of 20 days, and feed continuous. The anoxic zone had sizes ranging from zero (completely aerobic) through 39 and 50 to 70 percent of the total system volume. The underflow recycle was increased as the anoxic volume fraction was increased so that the denitrification potential of the anoxic zone was always exceeded and nitrate always was present in the effluent from the anoxic reactor. As the anoxic reactor volume fraction increased so the sludge settleability deteriorated. These large anoxic volumes could hardly be considered as selectors and very likely did not stimulate a rapid soluble biodegradable COD uptake rate, but they would have removed virtually all the soluble biodegradable COD. In terms of the selector hypothesis, because there was no selector effect, one would expect bulking with each of the anoxic volume fractions. Instead, bulking was absent in the 100 percent aerobic system and bulking increased progressively as the anoxic mass fraction increased. Unfortunately no filament identification was undertaken.

In the anoxic–aerobic work above, with systems that could reasonably be categorized as low F/M, either the filamentous organisms were not identified or where the filaments were identified, e.g. Lee *et al.* (1982), Shao (1986) and Still *et al.* (1986), the dominant filaments were not low F/M ones. Indeed, there appear to be no reports that unequivocally established that anoxic selectors or anoxic reactors control bulking by low F/M filaments. There is no doubt that the selector effect

an aerobic selector upstream of the main reactor (O/CFCM/SEL).

In terms of the selector hypothesis, the first (O/CFCM) should bulk, but the second and third (O/IFFD and O/CFCM/SEL) should not.

All systems were fed with unsettled sewage from a residential area. The systems were started with mixed liquor from a nitrification–denitrification plant serving the residential area. This plant produced a bulking sludge, DSVI > 250 mℓ/g with type 0092, *M. parvicella*, types 0675, 0041 and *Nocardia* filaments. The fill and draw system was fed once daily. Dissolved oxygen in the reactors was maintained greater than or equal to 2 mg/ℓ. All systems nitrified.

The fill and draw (O/IFFD) and the single reactor with the selector (O/CFCM/SEL) systems both developed a selector effect, that is, a high RBCOD uptake rate 2–3 times that from the single reactor constant feed (O/CFCM) system. The selector effect could be stimulated or lost over a period of less than one sludge age on changing the system from (1) to (2) or (3), or from (2) or (3) to (1) respectively.

The results were unexpected: *Irrespective of whether the systems developed the selector effect or not, the low F/M filaments present in the parent sludge disappeared and a well settling sludge developed.* In the O/CFCM system sporadic bulking was encountered due to the filament *S. natans*, a filament never observed to cause bulking in South African full–scale N and N & P removal systems. However, it was found that this was caused by seeding of the laboratory–scale system from attached growth on the walls of the feeding tube (Gabb *et al.*, 1989b). Twice weekly cleaning and chlorinating of the tubes resolved this seeding problem and subsequently no bulking due to *S. natans* was encountered. The seeding, therefore, was an artifact arising from laboratory–scale operation. In many of the aerobic laboratory–scale studies incorporating the selector (reviewed above), this artifact very likely was present causing bulking to be due to *S. natans* and not to low F/M filaments. As reported earlier this artifact had been observed previously at pilot–scale by Gabb *et al.* (1985).

The conclusion from this study was that bulking due to low F/M filaments did not occur in long sludge age (> 15 days) completely aerobic systems irrespective of whether a selector effect was present or not.

the NDBEPR system. From the survey of Blackbeard *et al.* (1988), the Carousel ND system appeared particularly prone to bulking by low F/M filaments. Accordingly, the following laboratory-scale system was set up to mimic the behaviour of the Carousel.

Two parallel single reactor systems were set up, both continuously fed and completely mixed, and operated at a sludge age of 20 days and at around 20°C. An unaerated-aerated pattern of 6–7 minutes anoxic and 3–4 minutes aerobic in a 10 minute cycle was set up. The large anoxic period was selected by noting that in Arkley and Marais' investigation, the SVI increased with the anoxic mass fraction.

In operating the plants, it was found very difficult to control the oxygen input to give the selected aerobic period of 3–4 minutes. The air on/air off period had to be found by trial and error. The period of aeration depended on the OUR, nitrification rate and MLSS. The air on/air off cycle eventually adopted was 55 seconds air on which increased the DO to approximately 2 mg/ℓ; over the next 2–3 minutes the DO decreased to zero and remained so until the next aeration cycle commenced. It was found that the OUR was dominantly affected by the ammonium concentration in the influent. In order to ensure a high OUR so that the DO would drop rapidly after aeration ceased, additional ammonia was added to the influent to stimulate a high nitrification OUR. The additional nitrate generated also ensured that denitrification did not reduce the nitrate concentration to zero during the anoxic period, (thereby ensuring anoxic, not anaerobic conditions), with about 3–5 mg (NO_3^--N)/ℓ) remaining at the end of the anoxic period. Throughout the test period, at intervals, anoxic and aerobic batch tests were performed; these showed that both systems did not have a selector effect.

In both systems the DSVI increased from about 110 to between 500 and 600 mℓ/g by day 103. On this day, in one unit intermittent aeration was replaced by continuous aeration – the DSVI decreased rapidly to about 50 mℓ/g. In the other unit with intermittent aeration, the DSVI rose to 600 mℓ/g due to proliferation of *M. parvicella*, and types 1851, 0092, 0914, all low F/M filaments. On day 174, the unit with continuous aeration was changed back to intermittent aeration and slowly the DSVI increased to over 300 mℓ/g by day 304. Meanwhile, from day 140 the unit that had intermittent aeration all the time also showed a decrease in DSVI, from 600 to stabilize at about 300 mℓ/g by day 300, but now the dominant filaments were 0092, 0914, *M. parvicella* and 0041. The two sludges were mixed and

In long sludge age systems,

(1) the selector effect appears to have no significant effect on the proliferation or suppression of low F/M filaments in both aerobic and alternating anoxic–aerobic systems,

(2) low F/M filaments will not proliferate in 100 percent aerobic systems,

(3) a *necessary* condition for low F/M filament proliferation is alternation between anoxic and aerobic states, however,

(4) alternation between anoxic and aerobic states is not a sufficient condition because some anoxic–aerobic systems bulk with low F/M filaments whereas others do not.

2.7 CLOSURE

The finding that the selector effect did not control low F/M filament bulking placed research in this field back into an exploratory stage. Accepting that anoxic–aerobic alternation is an essential condition to stimulate low F/M filament proliferation, enquiry into the following was considered necessary:

(1) Experimental investigation into the conditions leading to bulking in anoxic–aerobic systems; apart from anoxic–aerobic alternation other conditions may also have to be satisfied for low F/M filament proliferation. A systematic study of the filament response under separate aerobic and anoxic conditions, and in transition from aerobic to anoxic and from anoxic to aerobic conditions needs to be undertaken.

(2) The fact that both low F/M filaments and floc–formers are sustained in anoxic–aerobic systems implies that the species of both groups present in the sludge are facultative. Because anoxic–aerobic alternation is a necessary requirement for low F/M filament proliferation, it would indicate that a study of the behavioural patterns of facultative organisms operating under cyclic aerobic–anoxic changes of state may contribute to the understanding of the causes for the proliferation or reduction of these filaments.

CHAPTER 3

EXPLORATORY EXPERIMENTAL INVESTIGATIONS

ABSTRACT

Experimental work is described which investigates the causes of low F/M filament proliferation in system configurations in which sludge is exposed to alternating anoxic–aerobic conditions and fed defined artificial substrate and municipal sewage. A combination of conditions is identified which results in low F/M filament proliferation, namely, exposure of sludge to alternating anoxic–aerobic conditions with slowly biodegradable COD (SBCOD) present under conditions in which the aerobic mass fraction comprises 30 to 40 percent of the total and nitrite (NO_2^-) is present at a concentration greater than about 1,5 mgN/ℓ when conditions change from anoxic to aerobic.

3.1 INTRODUCTION

From the conclusions of the review into bulking in Chapter 2, eight aspects have been identified which possibly have an influence on proliferation of low F/M filamentous organisms. These eight aspects are as follows:

(1) Readily biodegradable COD (RBCOD) or slowly biodegradable COD (SBCOD) only as influent substrate.

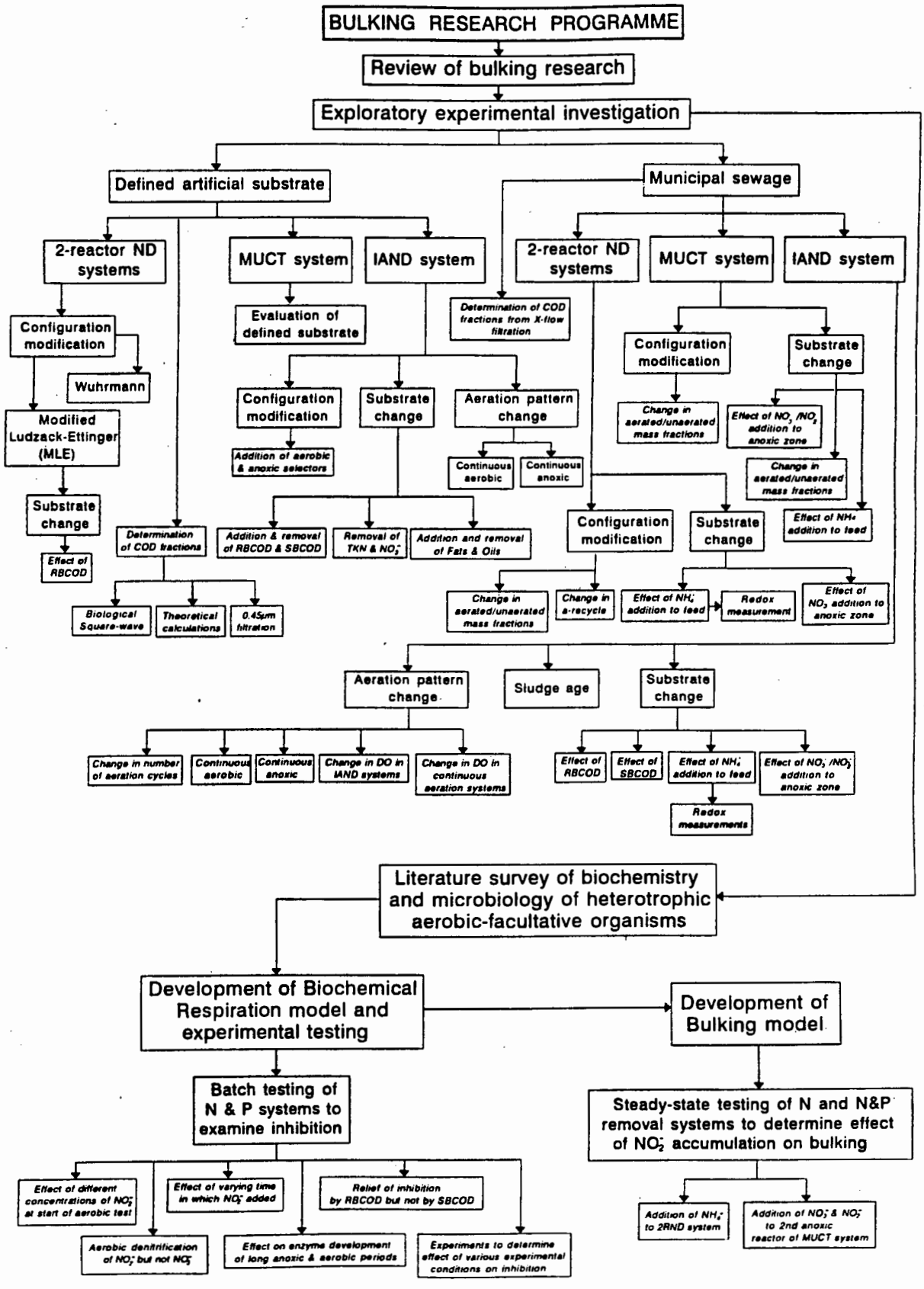
(2) continuous aerobic and continuous anoxic conditions.

(3) Magnitude of the aerobic mass fraction.

(4) Sludge age.

(5) Nitrate (NO_3^-) and nitrite (NO_2^-) concentrations in the anoxic zones.

(6) Differences between alternating anoxic–aerobic conditions caused by intermittent aeration conditions in a single reactor and separate 2–reactor anoxic–aerobic conditions.



Flowchart 3.1: Flowchart of the sequence of experimental and literature research conducted during the bulking research programme.

programme with defined substrate, experiments are described with a minimum of detail, with the exception of results which had a significant influence on the development of the hypothesis (for details see Appendix A).

Experimental results and discussion

Filamentous organisms in MUCT systems

Initial experimental investigations were conducted with an MUCT system (MUCTA) but due to an inability to develop a bulking sludge, experimental work with this configuration was abandoned and a configuration which would lend itself to proliferation of filamentous organisms was sought. (The details of the experiments with MUCTA are described in Appendix A). From the review of bulking it was established that IAND systems tend to produce bulking sludges and this configuration was adopted for the remainder of the experiments with defined substrate.

Effect of inclusion and removal of fats and oils in defined substrate composition on proliferation of M. parvicella

Three of the four IAND systems (Systems 1, 2 and 3) were examined for the effect of fats and oils on the proliferation of *M. parvicella*. In the sludges of the three systems *M. parvicella* was either absent or was present as a tertiary or secondary filament. The three systems were fed defined substrate in which fats and oils were either present or absent. Irrespective of whether fats and oils were present or absent in the substrate or the level at which *M. parvicella* was initially present in the sludge, it did not proliferate to become the dominant filament. Throughout the test periods in all systems, *Haliscomenobacter hydrossis (H. hydrossis)* was most often the dominant filament and types 1851 and 0092 most often the secondary filaments. It was concluded that either *M. parvicella* required a condition additional to fats and oils for proliferation, or that one of the constituents of the defined substrate provided a greater advantage to *H. hydrossis* and type 1851 than to *M. parvicella*. The changes in DSVI and filament type with time as a consequence of changes in substrate composition for Systems 1, 2 and 3 are shown in Figs 3.1, 3.2 and 3.3 respectively.

Inclusion and removal of RBCOD and SBCOD in the artificial substrate

Substrates containing either a large proportion of RBCOD (RBCOD–rich), or a large proportion of SBCOD (SBCOD–rich) were fed to IAND Systems 1 and 3.

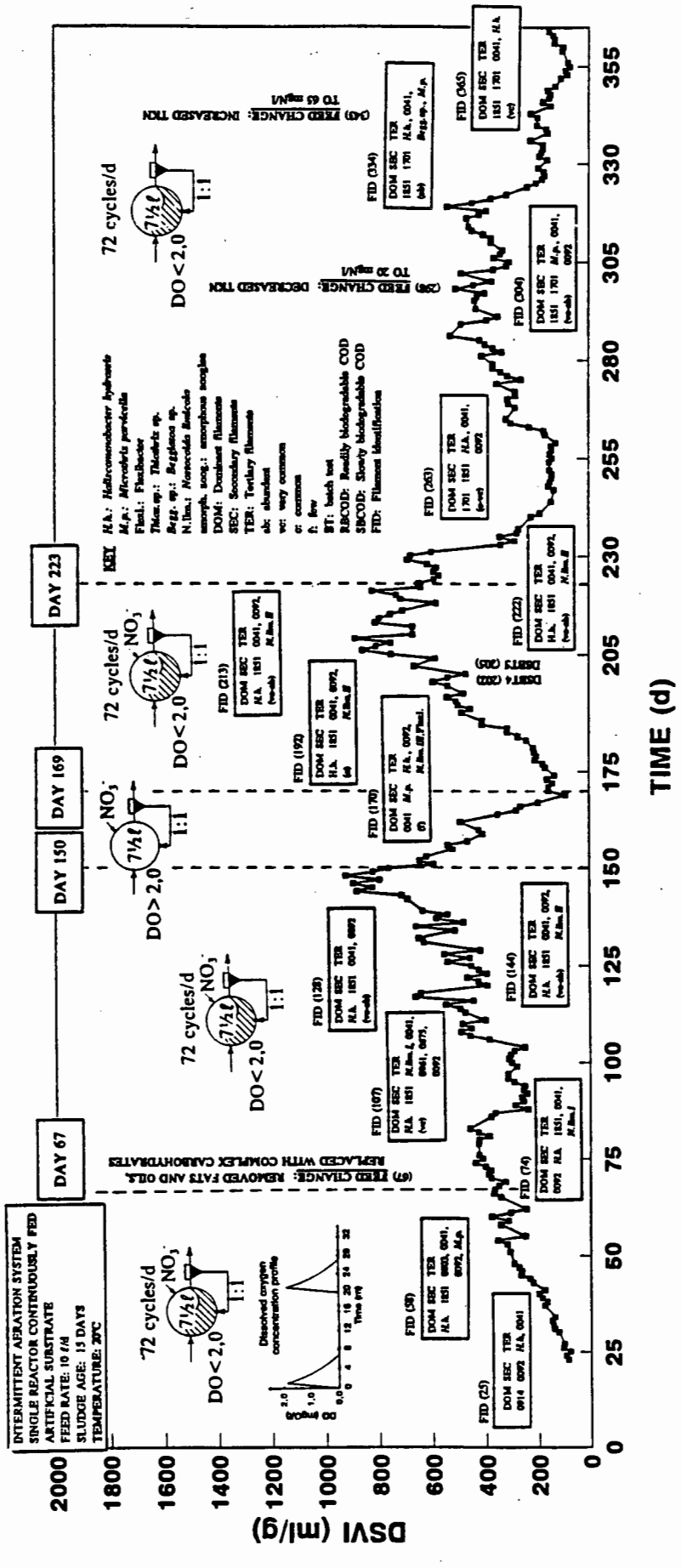


Fig 3.2: Sludge settleability as DSVI (ml/g) and changes in system operation with time (d) for System 2 fed defined artificial substrate.

The substrates to each system were changed twice; from SBCOD–rich to RBCOD–rich to SBCOD–rich substrate for System 1 and from RBCOD–rich to SBCOD–rich to RBCOD–rich substrate for System 3. For both systems, the DSVI increased substantially following a change from SBCOD–rich to RBCOD–rich substrate (from ≈ 500 to ≈ 1000 mℓ/g) and decreased similarly following a change from RBCOD–rich to SBCOD–rich substrate, the dominant filaments either *H. hydrossis* or type 1851, or both. The change in DSVI and filament type for Systems 1 and 3 with time during changes in substrate composition are shown in Figs 3.1 and 3.3 respectively.

An interesting result associated with the experiments concerns the change in combined NO_3^- and NO_2^- concentration (NO_{2+3}^-)[1] in the effluent with change in substrate. When the DSVI increased as a consequence of the addition of RBCOD–rich substrate, the effluent NO_{2+3}^- concentration decreased. Additionally, small daily changes in DSVI during periods of steady state operation resulted in corresponding small changes in NO_{2+3}^- concentration in the effluent. These changes are shown in Figs 3.4 and 3.5 for Systems 1 and 3 respectively.

Examination of the selector effect

The use of defined substrate of flexible composition provided an opportunity to test Chudoba's selection criteria for filamentous and floc–forming organisms by feeding the system RBCOD–rich substrate. According to the selection criteria, the addition of correctly sized selector reactors to a system with a bulking sludge fed RBCOD will control the proliferation of filamentous organisms – the high concentration of RBCOD in the selectors will favour the growth of floc–forming organisms and the substrate will be completely utilized. The selection criteria was examined with configurations incorporating aerobic and anoxic selector reactors and measurement of the selector effect with aerobic batch tests. Implicit to the selection criteria s that it is high substrate uptake rates (selector effect) induced by the high concentration of RBCOD which control filament proliferation, not the selector reactor configuration *per se*. Therefore, it can be concluded that irrespective of the means by which the selector effect is induced, filament proliferation can be controlled. It follows that the presence of high proportions of RBCOD in influent

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[1] The notation NO_{2+3}^- is used to denote the sum of the concentrations of NO_2^- and NO_3^-, in mgN/ℓ. Because the concentration of NO_2^- was not measured in these experiments, the individual concentrations of NO_3^- and NO_2^- could not be calculated.

fed to systems without selector reactors may be sufficient to induce the selector effect and control filament proliferation. Induction of the selector effect was examined with aerobic batch tests on sludge from systems fed substrate with different proportions of RBCOD.

Addition of aerobic and anoxic selectors: The fourth IAND system (System 4) which was fed the RBCOD–rich substrate, developed a DSVI of 1200 mℓ/g; the dominant filaments were *H. hydrossis* and type 1851. Addition to the system of two aerobic selectors comprising in total 12% of the final system volume reduced the DSVI from 1200 to 270 mℓ/g.[2] When the selectors were removed, the DSVI increased rapidly from 270 to more than 1500 mℓ/g; the dominant filament was *H. hydrossis.* Two anoxic selectors comprising in total 12% of the final system volume reduced the DSVI from nearly 1600 to 460 mℓ/g, but the addition of a third anoxic selector did not reduce the DSVI further. COD profiles conducted through the system when the aerobic and anoxic selectors were incorporated indicated that the major fraction of COD from the influent was removed in the 2 aerobic selectors (92%), 2 anoxic selectors (94%), and 3 anoxic selectors (97%). Figure 3.6 illustrates the changes in DSVI with time for System 4.

Comparison of the DSVI values of IAND System 4 fed RBCOD–rich substrate incorporating aerobic selectors (270 mℓ/g) and anoxic selectors (400 mℓ/g) with the DSVI values of IAND systems fed municipal sewage without selectors (250 mℓ/g) (see Warburton *et al.*, 1991) would indicate failure of selector reactors to control filament proliferation. However, as noted above, systems without selector reactors fed average defined substrate and RBCOD–rich defined substrate developed sludges with very high DSVI values (> 1200 mℓ/g) and far in excess of those associated with similar systems fed municipal sewage so on the basis of DSVIs in the 4 defined substrate systems, the relatively low DSVI values observed in System 4 with aerobic and anoxic selectors and fed RBCOD–rich substrate compared with systems fed RBCOD–rich substrate without aerobic and anoxic selectors indicated that the selector reactors controlled the filament proliferation. These results conform to Chudoba's selection criteria in which floc–forming organisms dominate as a result of their higher substrate uptake rates at high concentrations of RBCOD – with the floc–forming organisms taking up more than 92% of the influent substrate, there was

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[2] In order to maintain the percentage aerobic period of the intermittently aerated main reactor between 30 and 35 percent, the aerobic period was decreased upon the addition of the aerobic selectors and increased upon the addition of the anoxic selectors.

very little left for growth of filamentous organisms in the intermittently aerated main reactor. Aerobic batch tests to examine the selector effect were conducted on sludge from System 4 during periods when it incorporated 2 aerobic selectors, 2 anoxic selectors,and 3 anoxic selectors. For all configurations a selector effect was measured. This finding supports the selection criteria proposal, that high concentrations of substrate under aerobic or anoxic conditions induce high substrate uptake rates. The results and details of the batch tests are given in Appendix A.

RBCOD and SBCOD proportions in the substrate: Aerobic batch tests (DSBT3 – System 3 – Day 199; DSBT4 – System 2 – Day 202; DSBT10 – System 1 – Day 328) were conducted on sludge from three systems without selector reactors. The systems, the substrate fed to the systems, and the DSVIs of the systems were; System 3 (substrate of average RBCOD–SBCOD composition DSVI ≈ 300 mℓ/g), System 2 (SBCOD–rich substrate DSVI ≈ 600 mℓ/g), System 1 (RBCOD–rich substrate, DSVI ≈ 1500 mℓ/g). The substrates fed to the batch tests were all of average composition which contained sufficient RBCOD to measure a selector effect, if present. The results of the batch tests are plotted in Fig 3.7 and details of the batch tests are given in Appendix A.

System 1 with an excessively high DSVI exhibited a selector effect (high specific OUR) but Systems 2 and 3, also with high DSVIs, did not. From these results it can be concluded that high concentrations of substrate under aerobic conditions (irrespective of whether it is utilized in a selector reactor configuration) induce high substrate uptake rates. Further, with reference to the selector effect measured in selector reactor systems, in retrospect it cannot be concluded that the selector reactor configuration and not the RBCOD composition was the cause of the high substrate uptake rates. The results also support the conclusions of Chapter 2 concerning the selector effect and contradict the proposed selection criteria – filaments can proliferate in systems irrespective of whether or not a selector effect is induced.

Continuous aerobic and continuous anoxic conditions

Ketley *et al.* (1991) reported that continuous aerobic and continuous anoxic systems fed either defined substrate or municipal sewage developed low DSVI values (< 150 mℓ/g).

Similar results for continuous aeration were noted for System 2. From Day 67 to

149, System 2 (fed defined substrate of average composition) was intermittently aerated and high DSVIs developed (> 800 mℓ/g) (Fig 3.2). In order to control the prolific growth of the filamentous organisms (*H. hydrossis* and type 1851) the system was continuously aerated for 20 days from Day 150. The DSVI decreased from 800 to 100 mℓ/g over the 20 day period. Reimposition of intermittent aeration on Day 169 resulted in an increase in DSVI again from 100 to 800 mℓ/g over a period of 35 days as illustrated in Fig 3.2.

Magnitude of aerobic mass fraction

From the work discussed above, it can be concluded that systems which are continuous aerobic or continuous anoxic develop low DSVIs but IAND systems in which sludge alternates between anoxic and aerobic conditions and in which the aerobic period is 30 to 40% of the total, develop high DSVIs. The generality of this result was examined by determining the steady state DSVI values established at different aerobic mass fractions for an IAND system (System 2) fed a substrate of average composition.

The changes in DSVI with time during changes in aeration pattern and nitrate concentration for System 2 are shown in Fig 3.2. Figure 3.8 illustrates the relationship between steady–state DSVI and average percentage aerobic period for periods of steady–state operation. The results indicate that the highest DSVIs develop at 30 to 40% aerobic mass fraction – aerobic mass fractions greater than 40% and less than 30% result in lower DSVI values.

Nitrate and nitrite concentrations in the anoxic zone

A result associated with the investigations into the roles of RBCOD and SBCOD in filament proliferation was that increases and decreases in DSVI were accompanied by increases and decreases in NO_{2+3}^- concentrations in the system. This result focussed attention on the roles of NO_2^- and NO_3^- in filament proliferation although it was uncertain as to whether changes in NO_2^- and NO_3^- concentration caused, or were caused by, changes in filament proliferation.

Experiments with intermittently aerated System 2 with continuous NO_3^- addition (effluent $NO_{2+3}^- > 25$ mgN/ℓ) and high DSVI (> 600 mℓ/g) indicated that when NO_3^- addition was stopped (effluent $NO_{2+3}^- < 5$ mgN/ℓ), the DSVI decreased from more than 600 mℓ/g to below 150 mℓ/g in 20 days (see Fig 3.2, Days 223 to 257); the decrease being due to a reduction in *H. hydrossis* growth. However, in the

continued absence of added NO_3^-, the DSVI increased, from less than 150 mℓ/g to 400 mℓ/g over a period of 40 days (Days 258 to 298) through the proliferation of filament types 1851 and 1701. Removal of the ammonium (NH_4^+) fraction of the substrate (an organic nitrogen fraction was available for cell growth) on Day 298 in order to restrict nitrification, (effluent $NO_{2+3}^- < 1$ mgN/ℓ) resulted in a decrease in DSVI from 400 to less than 200 mℓ/g over a 44 day period (Days 298 to 342); filament types 1851 and 1701 demonstrating reduced proliferation. The concentration of NO_{2+3}^- in IAND systems appears to have had a significant effect on filament proliferation, but different concentrations of NO_{2+3}^- affect different filament types to different extents; it appears that under intermittent aeration conditions, *H. hydrossis* requires higher NO_{2+3}^- concentrations (> 5 mgN/ℓ) than filament types 1851 and 1701 (< 5 mgN/ℓ) in order to proliferate.

Differences in sludge settleability between IAND and 2RND systems with apparently similar operating conditions

From the work conducted with IAND Systems 1–4 above, it is apparent that filaments proliferate with (1) RBCOD or SBCOD, under (2) alternating anoxic–aerobic conditions, and (3) the presence of NO_3^- and NO_2^- exacerbates the proliferation (DSVI ≈ 400–600 mℓ/g). In contrast to this Hulsman *et al.* (1992) found that for 2RND configurations fed the same defined substrate composition, and with an aerobic mass fraction between 30 and 40% of the total, the usual filaments such as *H. Hydrossis* and 1851 did not proliferate to the same degree (DSVI ≈ 150–200 mℓ/g). The difference in filament proliferation between sludges in the IAND and the 2RND systems, with apparently similar exposure to aerobic and anoxic conditions was noteworthy and is investigated further in Part II below.

Conclusions

From the results of the investigations described above, the following observations and conclusions can be made:

(i) *M. parvicella* did not proliferate under intermittent aeration conditions fed artificial substrate, irrespective of the presence or absence of fats and oils in the substrate.

(ii) *H. hydrossis* and type 1851 proliferated in intermittently aerated systems fed either RBCOD–rich substrate (DSVI ≈ 900 mℓ/g) or SBCOD–rich substrate (DSVI ≈ 500 mℓ/g) or average substrate (30/70 RBCOD/SBCOD

differences were noted:

- The frequency of exposure of sludge to alternating anoxic–aerobic cycles in an IAND system is high (> 30/d) as a result of the large number of intermittent aeration cycles per day, but for a 2RND system is low (< 5/d) as a consequence of the low a– (acrobic–anoxic) and s– (sludge) recycles.

- The RBCOD and SBCOD fractions of the influent are fed into both the aerobic and anoxic periods in IAND systems but in 2RND systems, the influent is all fed into the anoxic zone only (for pre–denitrification MLE systems) or into the aerobic zone only (for post–denitrification Wuhrmann systems).

- The DO concentration in the aerobic period of an IAND system changes progressively from a high value (DO ≈ 2,0 mgO/ℓ) to zero, passing through a period of low DO as a result of biological action, but in a 2RND system the DO concentration remains constant in the aerobic reactor (DO > 2,0 mgO/ℓ) and changes relatively quickly to zero in the anoxic reactor.

- The NO_2^- and NO_3^- concentrations in the anoxic period of an IAND system decrease as a result of denitrification, but in a 2RND system at steady–state, the NO_2^- and NO_3^- concentrations remain constant in the anoxic reactor.

These four differences between IAND and 2RND systems were tested experimentally with municipal sewage as influent and the results are described in Part II of this Chapter.

After operating IAND Systems 1 to 4 for a period of a year (365 days) the use of defined artificial substrate was discontinued for two reasons. The first reason is that the primary objective in its use was fulfilled, i.e. to investigate whether or not filament proliferation can take place on a SBCOD–rich as substrate. The second reason is that the filaments which dominated the four laboratory systems fed the defined substrate *H. hydrossis*, type 1851 and type 1701 were not the same filaments found in full–scale N and N & P removal systems, (*M. parvicella*, type 0092 and type 0914). Real municipal sewage was used in all further experimental work.

Because of the importance of conducting laboratory–scale experiments with the same filamentous organisms as are found in full–scale systems, many of the aspects

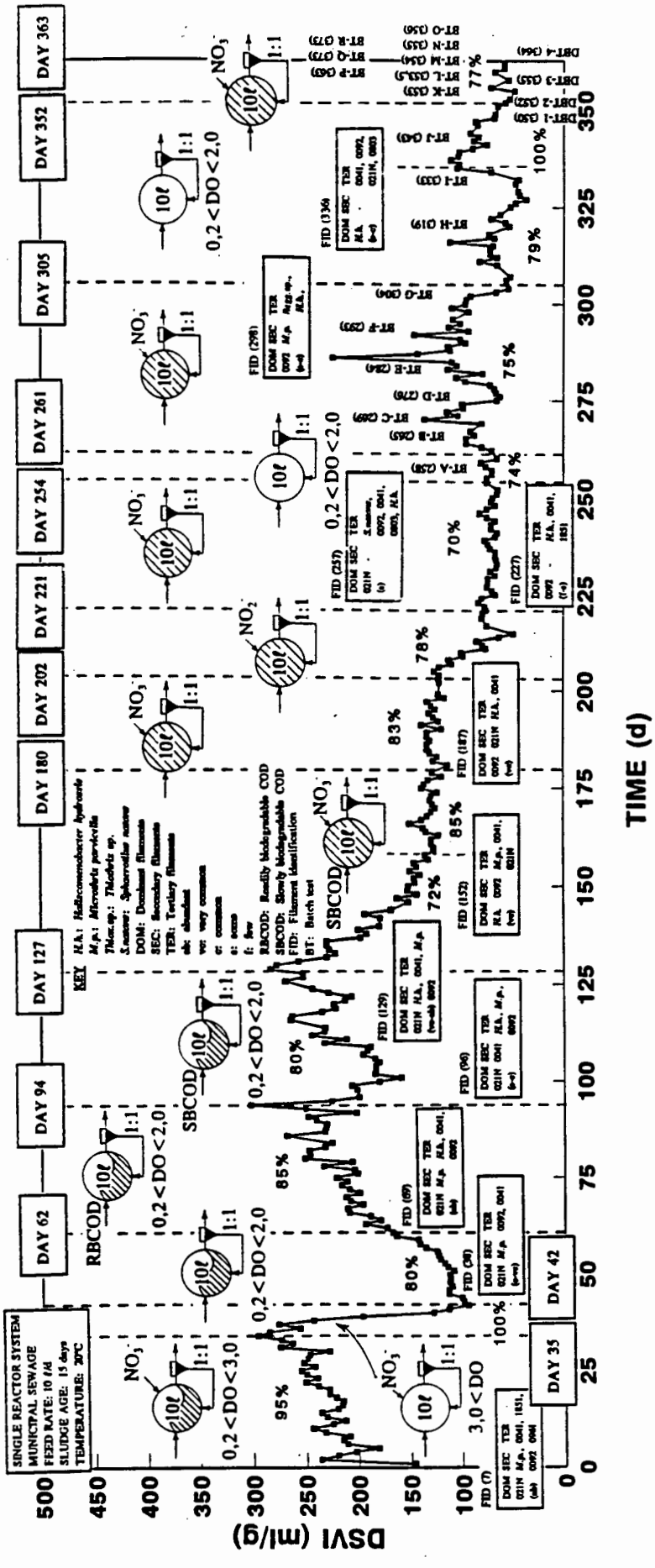

Fig 3.11: Sludge settleability as DSVI (mℓ/g) and changes in system operation with time (d) for System 7. (Percentage values refer to COD mass balances).

just over a sludge age. For each of Systems 5 to 8, the rapid reduction in DSVI with continuous aeration was associated with an increase in the COD balance. Under continuous aeration, the COD balance was between 98 and 100 percent, but with intermittent aeration in which the DO ranged between 0,2 and 3,0 mgO/ℓ, the COD balance was 86 to 95% and with intermittent aeration in which the DO ranged between 0,2 and 3,0 mgO/ℓ, the COD balance was between 73 and 81 percent.

It was found also that other periods during which conditions were changed to continuous aerobic from intermittent aeration (System 5, Days 62 to 94; System 6, Days 95 to 153), the COD balance improved from around 75 percent to between 95 and 105 percent. Reasons for the improvement in COD balance from intermittent aeration conditions (under which a high DSVI developed) to continuous aeration (under which a low DSVI developed) are proposed in Chapter 6 in which a biochemical/microbiological model for bulking is developed. Ketley *et al.* (1991) also investigated the effect of continuous aerobic conditions on bulking sludges developed in three IAND systems. In each system, *M. parvicella* proliferation in the sludge as a consequence of intermittent aeration conditions resulted in a DSVI ≈ 400 mℓ/g, and changing the aeration pattern from intermittent (30–35% aerobic) to continuous caused amelioration of filament proliferation (DSVI ≈ 100 mℓ/g). This result is in agreement with the results of Gabb *et al.* (1989a) in which *M. parvicella* proliferation in IAND systems fed municipal sewage was controlled by continuous aeration.

Continuous anoxic conditions: Ketley *et al.* (1991) investigated the effect of continuous anoxic conditions on bulking sludges developed in three IAND systems. Following the imposition of continuous anoxic conditions with excess NO_3^- supplied as electron acceptor, the DSVI decreased from ≈ 200 to ≈ 89 mℓ/g; the filaments *M. parvicella*, type 1851 and type 0092 each of which was dominant under intermittent aeration conditions, were controlled by continuous anoxic conditions.

Variation in aerobic mass fraction: In nitrification–denitrification (ND) systems, whether IAND, 2RND or ND biological excess phosphorus removal (NDBEPR) systems, the aerobic period serves two functions; (i) to supply oxygen as electron acceptor during heterotrophic organism utilization of organic matter and (ii) to supply oxygen for nitrification of influent NH_4^+ to NO_2^- and NO_3^- by autotrophic organisms as the preliminary step to nitrogen removal through denitrification. Warburton *et al.* (1991) examined the effect on filament proliferation of variation in

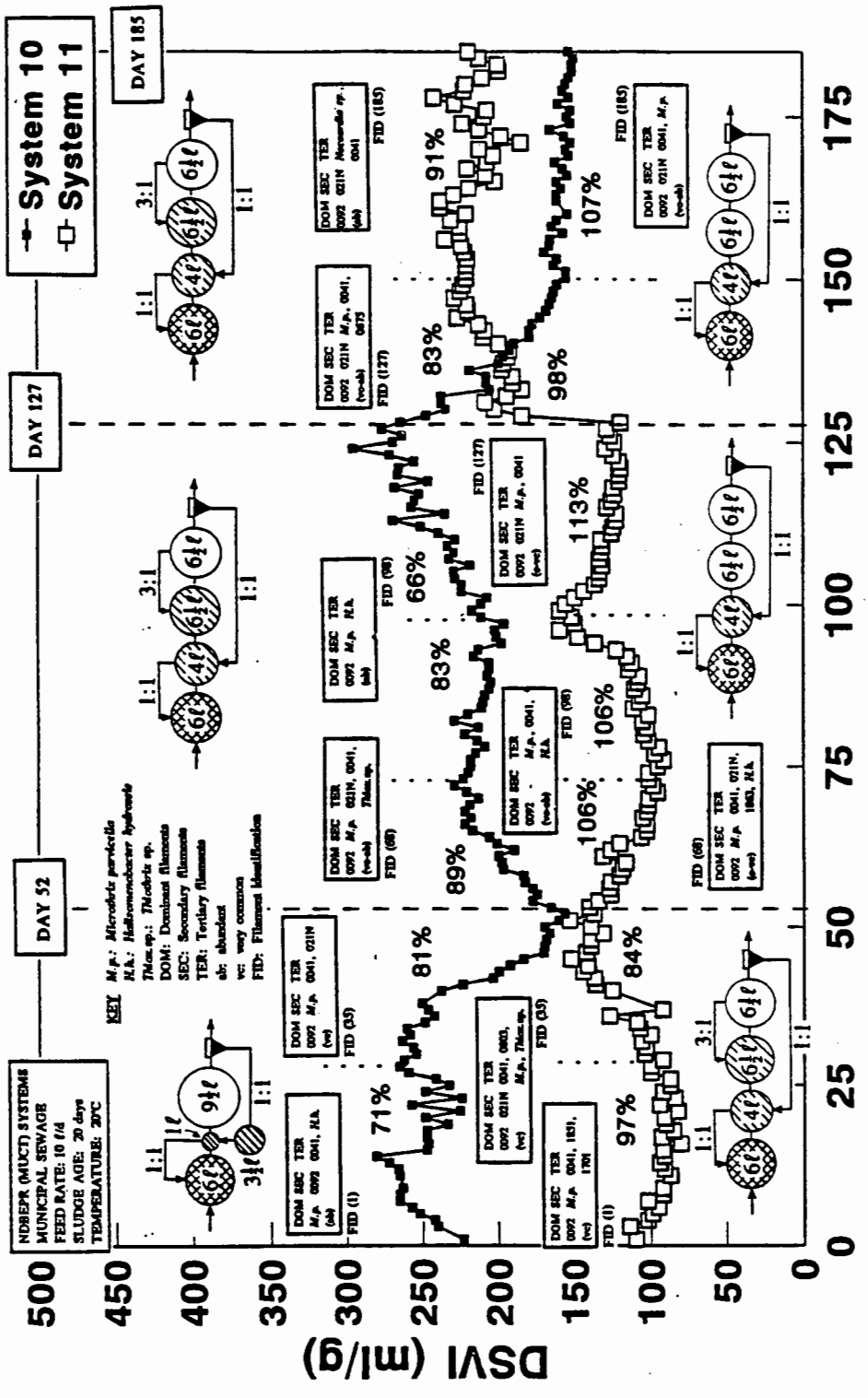

Fig 3.13: Sludge settleability as DSVI (mℓ/g) and changes in system operation with time (d) for MUCT Systems 10 and 11. (Percentage values refer to N mass balances).

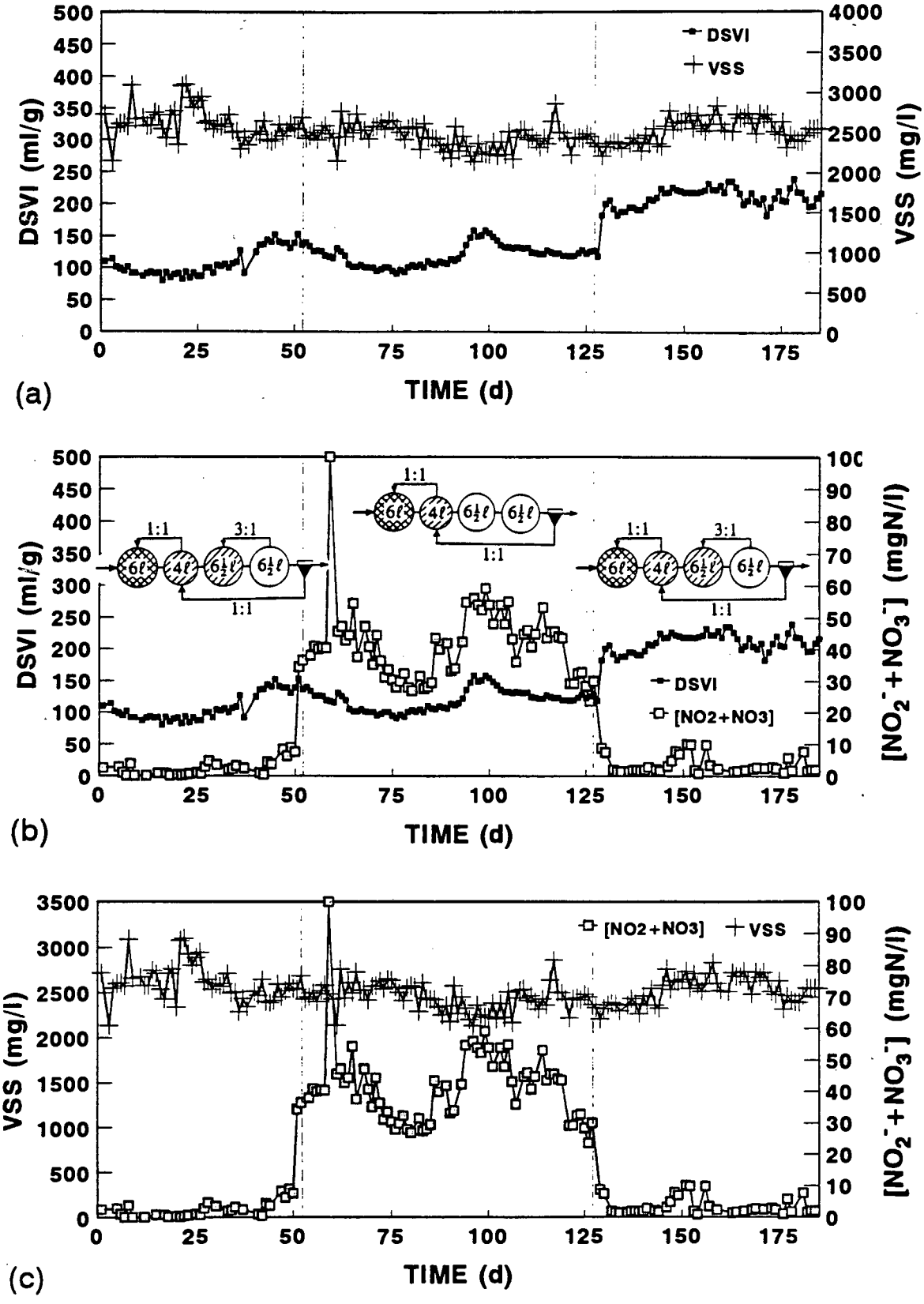

Fig 3.15: (a) DSVI and VSS with time, (b) DSVI and 2nd anoxic reactor (NO_2^- + NO_3^-) concentration with time, and (c) VSS and 2nd anoxic reactor (NO_2^- + NO_3^-) concentration with time for System 11.

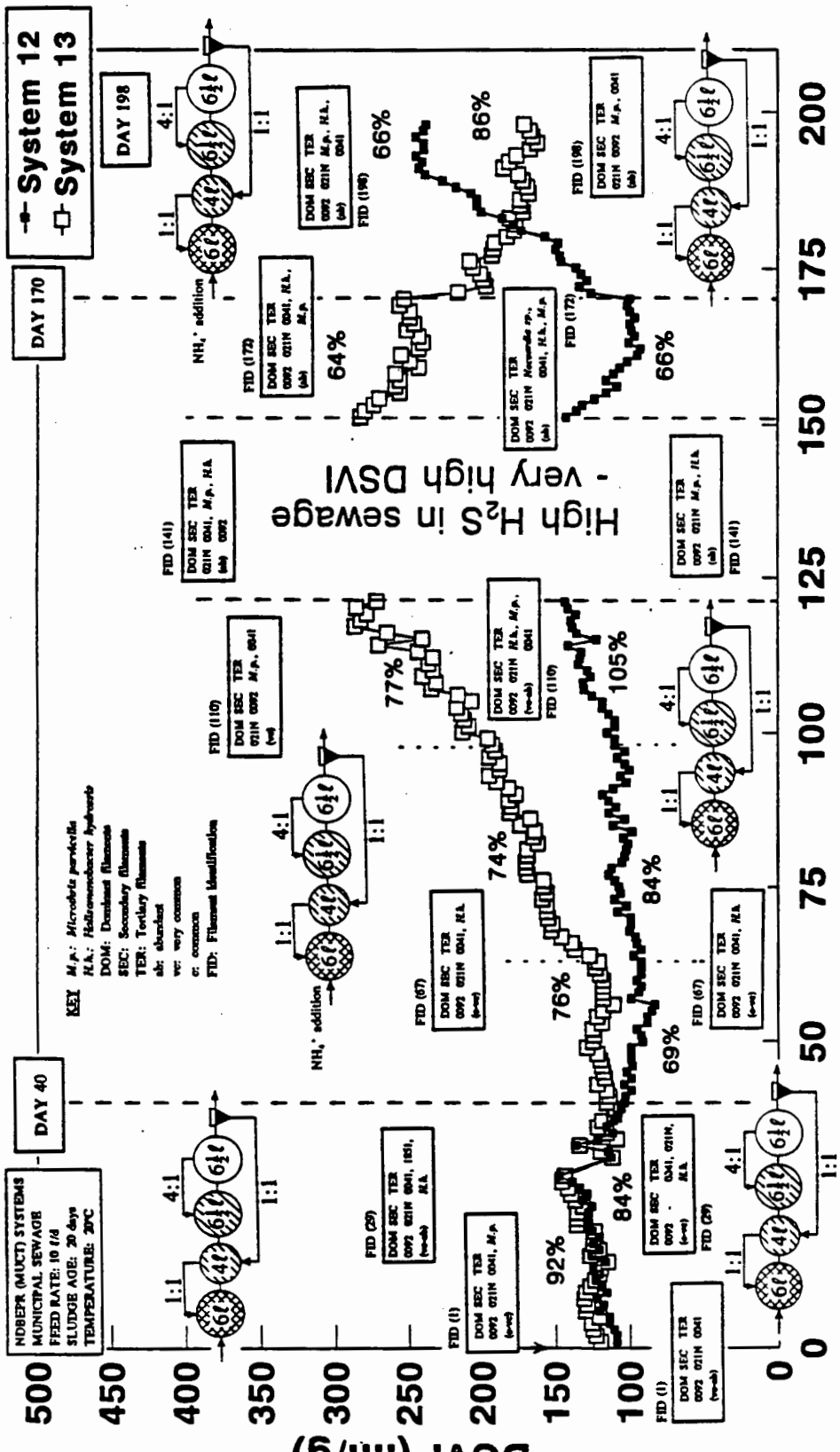

Fig 3.16: Sludge settleability as DSVI (mℓ/g) and changes in system operation with time for MUCT Systems 12 and 13. (Percentage values refer to N mass balances).

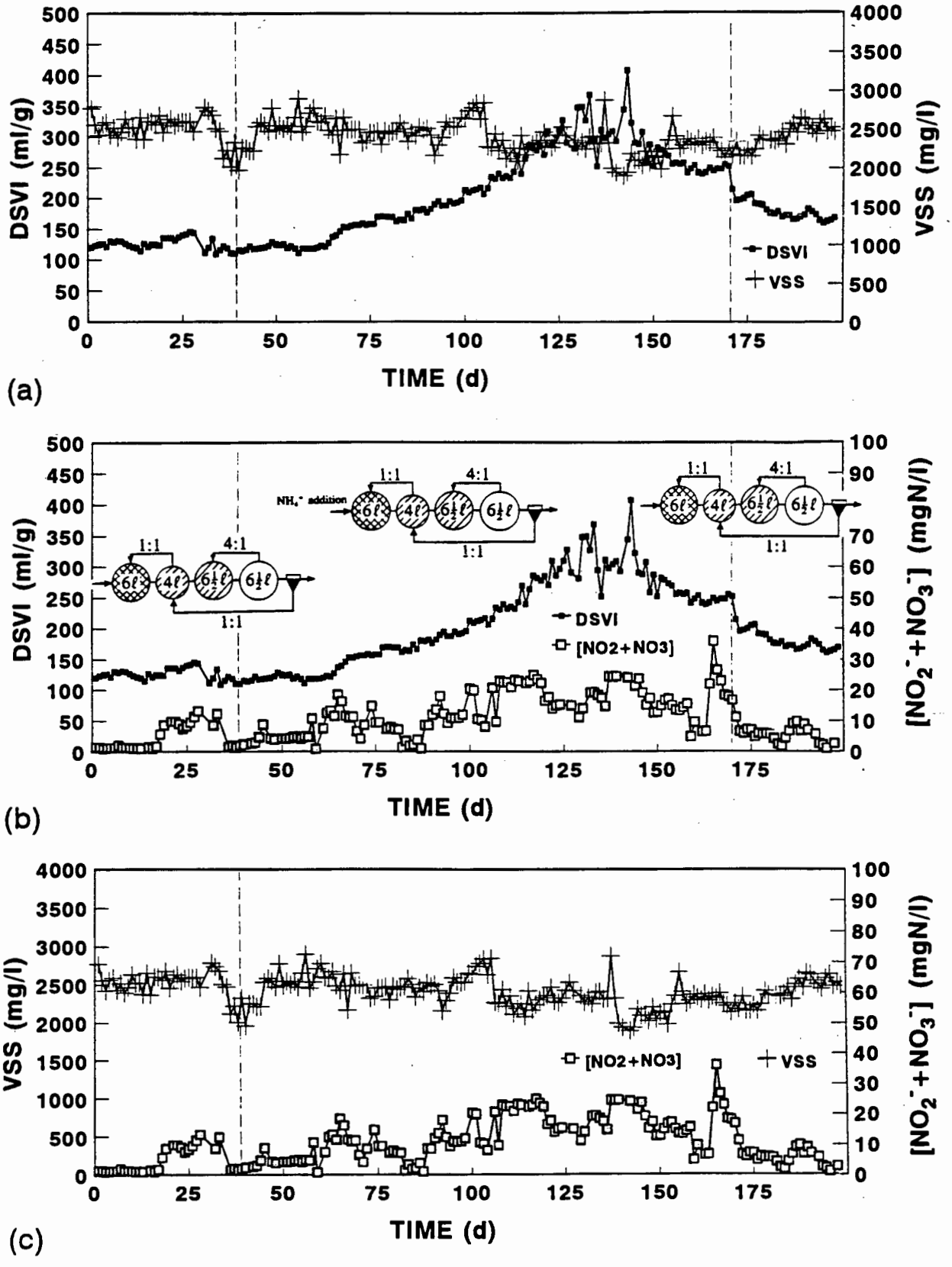

Fig 3.18: (a) DSVI and VSS with time, (b) DSVI and 2nd anoxic reactor (NO_2^- + NO_3^-) concentration with time, and (c) VSS and 2nd anoxic reactor (NO_2^- + NO_3^-) concentration with time for System 13.

configurations could play a role in filament proliferation, the mechanism by which this could occur was unclear.

(1) *Frequency of exposure of sludges to aerobic/anoxic conditions:*
The first difference concerns the frequency of exposure of sludges in IAND and 2RND configurations to aerobic/anoxic conditions. In IAND systems, each anoxic–aerobic cycle is about 20 to 30 minutes in length, which equates to between 72 and 48 cycles/day. In 2RND systems, the mixed liquor (a–) and underflow sludge (s–) recycle ratios determine the frequency of alternation, and in laboratory–scale systems normal operating conditions would be a = 1 to 3 and s = 1, i.e. the number of anoxic–aerobic cycles varies between 3 and 5/day, which is significantly lower than the 48 to 72 cycles/day in an IAND system. It was proposed that if filaments gain a benefit from exposure to alternating anoxic–aerobic cycles, then that benefit would be proportionally increased by exposure to increased numbers of anoxic–aerobic cycles.

To determine the effect of frequency of exposure of sludge to anoxic conditions, experiments were conducted both with IAND and 2RND systems.

- Ketley *et al.* (1991) examined IAND systems with aerobic periods of approximately 30% and anoxic–aerobic cycle lengths varying between 20 minutes (14 min anoxic, 6 min aerobic) and 3 days (2 days anoxic, 1 day aerobic). All systems had NO_3^- addition during the anoxic period and all systems developed bulking sludges (DSVI > 150 mℓ/g).

- Hulsman *et al.* (1992) examined 2RND systems with aerobic mass fractions comprising 30–40% of the total, one system with a low (3:1) a–recycle ratio, the other system with a high (>30:1) a–recycle ratio. The objective of the high a–recycle ratio in the 2RND system was to provide a frequency of exposure of the sludge to anoxic–aerobic conditions similar to IAND systems. The systems with the low and the high a–recycle ratios both developed non–bulking sludges (DSVI < 150 mℓ/g).

The results of Ketley *et al.* (1991) and Hulsman *et al.* (1992) indicated that the frequency of exposure of sludge to anoxic–aerobic conditions does not influence the proliferation of low F/M filaments; IAND systems bulked irrespective of the number of anoxic–aerobic cycles, and 2RND systems did

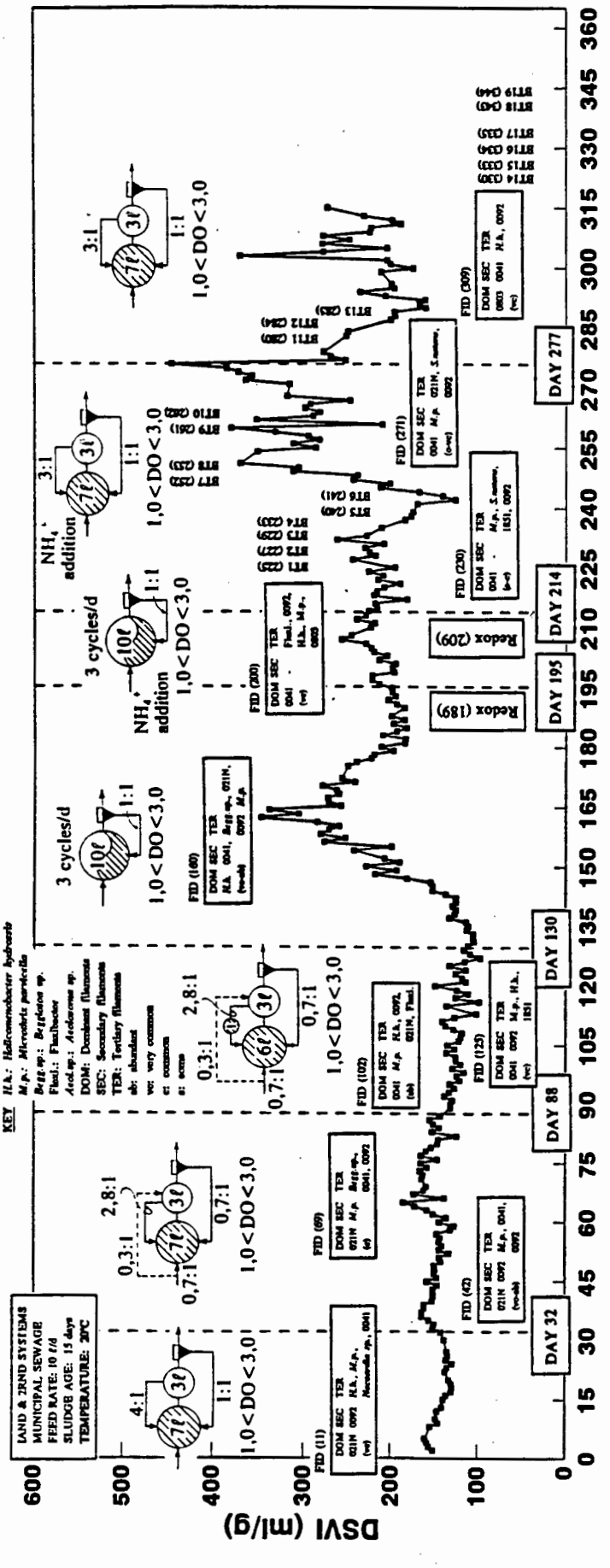

Fig 3.19: Sludge settleability as DSVI (mℓ/g) and changes in system operation with time (d) for System 9.

reactors in which the sludge is exposed to a low, or decreasing DO concentration.

To examine the effect of low DO conditions two experiments were conducted:

- Two single–reactor 15 day sludge age systems (Systems 5 and 6) were operated. System 5 had continuous low DO ($0,2 < DO < 0,5$) for 2 sludge ages (Days 61 to 93), and then intermittent aeration (35% aerobic, 65% anoxic) for 3 sludge ages (Days 94 to 149), the aerobic part of the cycle maintained at low DO ($0,2 < DO < 0,5$). System 6 had intermittent aeration for 2 sludge ages (Days 60 to 93), and then continuous low DO for 3 sludge ages (Days 94 to 152), the DO concentration the same as for System 5. The changes in DSVI and aeration pattern for Systems 5 and 6 are shown in Figs 3.9 and 3.10 respectively from which it can be seen that with continuous aeration at low DO, the DSVI was low and with intermittent aeration with low DO in the aerobic period the DSVI was high. The filamentous organisms causing the high DSVI were types 0092 and 0041, for System 5 and type 021N for System 6. From the results of the experiments it was concluded:

  (i) Filamentous organisms do not proliferate under continuously aerated conditions irrespective of whether the concentration of DO is low ($0,2 < DO < 0,5$), or high ($0,2 < DO < 1,0–2,0$).

  (ii) In order to proliferate, filamentous organisms require the presence of an anoxic period.

  (iii) For intermittent aeration conditions (35% aerobic, 65% anoxic) the higher the peak DO concentration during the aerobic period, the greater the proliferation of filamentous organisms.

  (iv) For continuous aeration conditions, the higher the DO concentration the more rapid the reduction in DSVI.

To examine the effect of a decreasing DO concentration in a 2RND system:

- A 2RND system (System 9) was operated with aerated and unaerated mass fractions of 30 and 70% respectively with the feed split between the aerobic and anoxic periods in proportion to the respective mass fractions of the aerobic and

electron acceptors. That is, under aerobic conditions, when O_2, NO_3^- and NO_2^- are present, the redox potential is high, and the first choice electron acceptor, O_2 is utilized. As oxygen is consumed, the redox potential decreases and the organisms switch to the use of the second–, and third–choice electron acceptors, NO_3^- and NO_2^- respectively. As each of these is consumed, the redox potential decreases until anaerobic conditions (no O_2, NO_3^- or NO_2^-) are reached.

With this principle as a gauge, a major difference is apparent in the redox potential of comparable zones in IAND and 2RND systems. For IAND systems, the scenario described above is applicable, but for 2RND systems, organisms experience a different set of redox conditions. A constant redox potential is established in each of the anoxic and aerobic reactors and the organisms, in moving between the two zones, experience a large and sudden change in redox potential.

To examine the differences in redox potential between IAND and 2RND systems the redox potential (E_h) and NO_3^- and NO_2^- concentrations were measured in the anoxic and aerobic reactors of a 2RND system and throughout the anoxic–aerobic cycle of an IAND system.

- Redox potential was measured in the anoxic and aerobic reactors of System 9 (see Fig 3.19) between Days 88 and 130. In the anoxic zone, the average value was –81 mV and in the aerobic zone, +48 mV.

- After establishing the above redox potentials, System 9 (DSVI ≈ 120 mℓ/g) was changed to an IAND system on Day 130. The DSVI increased over an initial period of 17 days to 300 mℓ/g before decreasing to and stabilizing at a DSVI of 180–200 mℓ/g by Day 195. On Day 189, redox measurements were made throughout one 8 hr intermittent aeration cycle and the concentrations of NO_3^- and NO_2^- were measured at intervals of approximately 20 minutes during the same cycle and are illustrated in Fig 3.20.

Following the introduction of oxygen at the start of the aerobic period the value of E_h increased from –130 mV and attained a value of +40 mV at the end of the $2^1/_4$ hrs aerobic period. During the same period the concentration of NO_2^- increased from zero to > 2,0 mgN/ℓ after 80 minutes due to nitrification of NH_4^+ to NO_2^- by *Nitrosomonas* and then decreased to 0,6 mgN/ℓ at the end of the aerobic period as a result of nitrification of NO_2^- to NO_3^- by *Nitrobacter*. The concentration of NO_3^-

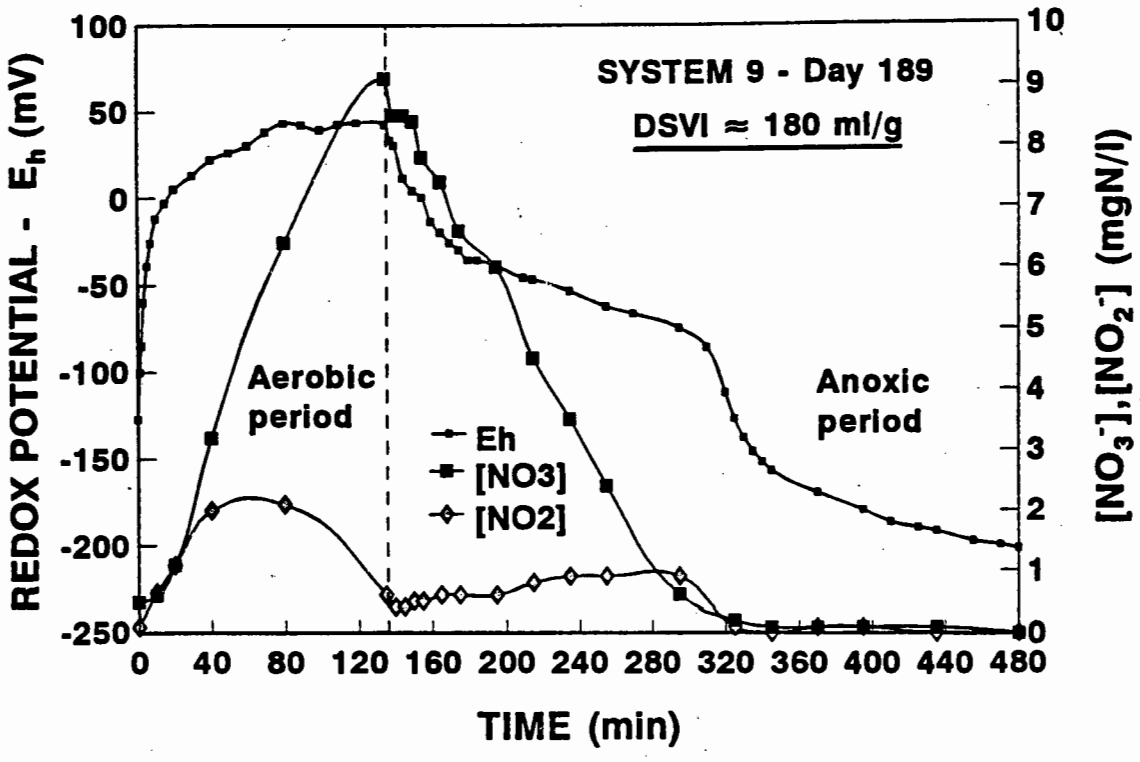

Fig 3.20: Redox potential (E_h in mV) with time (h) for an 8 h aerated–unaerated cycle of System 9 on Day 189.

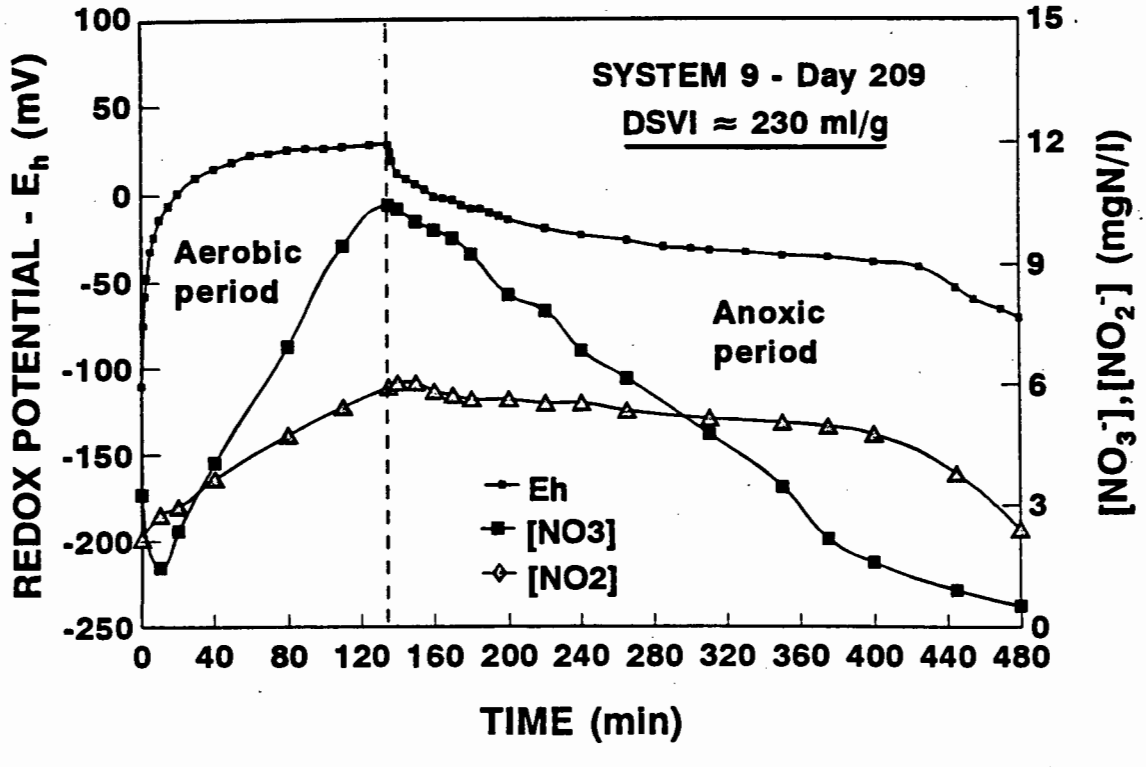

Fig 3.21: Redox potential (E_h in mV) with time (h) for an 8 h aerated–unaerated cycle of System 9 on Day 209.

variations in DSVI, also resulted in variation in the concentration of NO_{2+3}^- in the 2nd anoxic reactor, the outflow of which enters the aerobic reactor.

The focus of the investigation then changed from examining the relationship between redox potential and filament proliferation to examining the relationship between the NO_3^- and NO_2^- concentrations and filament proliferation. The first task was to determine, *if* the presence of NO_3^- or NO_2^- is the cause of filament proliferation, firstly, which of the two, NO_3^- or NO_2^- is responsible, and secondly, in which zone, anoxic or aerobic, do the filaments proliferate? This is a difficult task since in anoxic–aerobic systems one nitrogen oxide is always present as a result of the other, through either nitrification under aerobic conditions (i.e. $NO_2^- \rightarrow NO_3^-$), or nitrate reduction under anoxic conditions (i.e. $NO_3^- \rightarrow NO_2^-$). Therefore, as a preliminary and more simple task, the effect of NO_3^- and NO_2^- on filament proliferation was examined under fully anoxic conditions, i.e.

- Ketley *et al.* (1991) reported that under continuous anoxic conditions with the addition of excess NO_3^- as electron acceptor, the low F/M filaments did not proliferate. This confirmed that anoxic–aerobic alternation was required. In these experiments NO_2^- and NO_3^- concentrations were not measured individually, but this was not regarded as serious because the filaments did not proliferate.

- System 7 (a continuous anoxic system with excess NO_3^- continuously dosed) was fed only the SBCOD fraction of municipal sewage between Days 128 and 181 (see Fig 3.11). The DSVI at Day 181 was 125 mℓ/g and the system feed was changed from the SBCOD fraction to the complete municipal sewage (i.e. non–fractionated municipal sewage including both the SBCOD and RBCOD fractions). Between Days 181 and 203, the DSVI remained between 120 and 130 mℓ/g. The concentrations of NO_3^- and NO_2^- in the reactor were 30–40 mgN/ℓ and 1–3 mgN/ℓ respectively (NO_2^- was present as a result of the denitrification of NO_3^-). On Day 187 the dominant filament was type 0092. On Day 203, NO_3^- dosing was switched to NO_2^- dosing while the feed remained the complete municipal sewage. The DSVI decreased from 120 mℓ/g to 75 mℓ/g between Days 203 and 222. During this period, the concentration of NO_2^- in the reactor was about 40 mgN/ℓ and no NO_3^- was measured. On Day 222, the continuous NO_2^- dosing was switched to and NO_3^- again. The DSVI remained between 60 and 70 mℓ/g between Day 222 and Day 255 and the concentrations of NO_2^- and NO_3^- in the reactor were < 10 and ≈ 50 mgN/ℓ respectively.

At this point it is not the intention to evaluate all this information for its implications regarding the specific conditions required by individual filament types for proliferation, as this was not the intention of the exploratory investigation (such an analysis is conducted in Appendices A and B). Rather, the objective of the investigation was to eliminate the factors which have only a minor or negligible influence on low F/M filament bulking and to clarify the areas that have a major influence, thereby highlighting the direction that the research investigation should take to establish the cause(s) of the low F/M filament bulking problem.

In conformity with this objective the results of the investigation which apparently have a *major* influence on low F/M bulking are as follows:

1) Continuous aerobic conditions control filament proliferation to low DSVI values ($\approx$ 100 mℓ/g).

2) Continuous anoxic conditions control filament proliferation to low DSVI values ($\approx$ 100 mℓ/g).

3) Alternating anoxic–aerobic conditions in which the aerobic mass fraction is between 30 and 40 percent result in maximum proliferation of filaments; aerobic mass fractions increasingly less than 30 percent and increasingly more than 40 percent result in increasingly lower DSVI values.

4) Filamentous organisms proliferate in systems with sludges exposed to alternating anoxic–aerobic condition whether in IAND, 2RND or MUCT systems in which nitrate and/or nitrite are present throughout the anoxic period (IAND) or in the anoxic reactor just prior to the aerobic reactor (2RND, MUCT) i.e. nitrate and/or nitrite are present at concentrations exceeding 5 and/or 1 mgN/ℓ respectively, when the sludge is exposed to aerobic conditions.

5) With regard to the relative importance of nitrate and/or nitrite on filament proliferation in (4) above, nitrite appears to have a greater influence then nitrate on promoting filament growth.

6) Subject to the conditions (3) and (4) above, low F/M filaments proliferated irrespective of the biodegradability (RBCOD or SBCOD) of the substrate available.

a level more fundamental than the externally measured manifestations of activated sludge processes such as COD removal, and oxygen and nitrate uptake rates. This new direction entailed examination of the biochemical mechanisms of transport of protons, electrons, and the electron acceptors, oxygen, and nitrate and nitrite, and the synthesis of enzymes mediating these mechanisms under aerobic, anoxic, and alternating anoxic–aerobic conditions. Investigations in this direction were also prompted through having exhausted all areas of experimental research considered to be influential in filamentous organism bulking. Accordingly, an in–depth review of these biochemical aspects was conducted on obligate and facultative heterotrophic organisms and is presented in the next Chapter.

From the results of the experiments described in this Chapter it is apparent that the so–called low F/M filaments do not proliferate under all low F/M conditions; in particular, under continuous aerobic and continuous anoxic conditions, low F/M filaments do not develop. As a consequence of the finding that this group of filaments invariably proliferate under alternating anoxic–aerobic conditions, it was concluded that the filaments should be renamed anoxic–aerobic (AA) filaments, and this designation is used throughout the remainder of this investigation.

CHAPTER 4

BIOCHEMISTRY OF HETEROTROPHIC RESPIRATORY METABOLISM

ABSTRACT

Biochemical mechanisms for respiration and regulation of respiration for facultative organisms are reviewed in two sequential parts. In Part I, the biochemical reactions involved in the utilization of substrate under aerobic and under anoxic conditions are outlined. In Part II, the mechanisms which initiate, regulate, and terminate respiration under each of aerobic and anoxic conditions are described. A consequence of Part II is identification of a series of conditions and mechanisms in which one of the intermediates of denitrification (nitrite, or nitric oxide) interacts with the aerobic respiration enzymes (cytochrome oxidase), resulting in inhibited aerobic respiration under conditions in which organisms are alternately exposed to anoxic and aerobic conditions. This mechanism is an important aspect of a conceptual biochemical model for aerobic respiration developed in the next Chapter.

PART I: AEROBIC AND ANOXIC RESPIRATION IN FACULTATIVE ORGANISMS

4.1 INTRODUCTION

In the previous Chapter it was concluded that a new research direction is required for filamentous organism bulking research. As a starting point, it was considered that a more fundamental understanding is required of the biochemical mechanisms involved in respiration by aerobic facultative organisms. Consequently a literature review is conducted, the objective of which is to identify the principal electron transport pathways (ETPs) employed by facultative organisms under aerobic, anoxic, and alternating anoxic-aerobic conditions. Although the mechanisms of aerobic ETPs are well established for obligate aerobic organisms, the literature is somewhat piecemeal with regard to the mechanism of anoxic respiration, and considerably incomplete with regard to the mechanisms of respiration of facultative organisms, exposed to alternating anoxic–aerobic conditions. In this review it is the intention to ameliorate this situation by presenting an overview of current knowledge regarding the electron transport complexes and processes associated with facultative heterotrophic organisms.

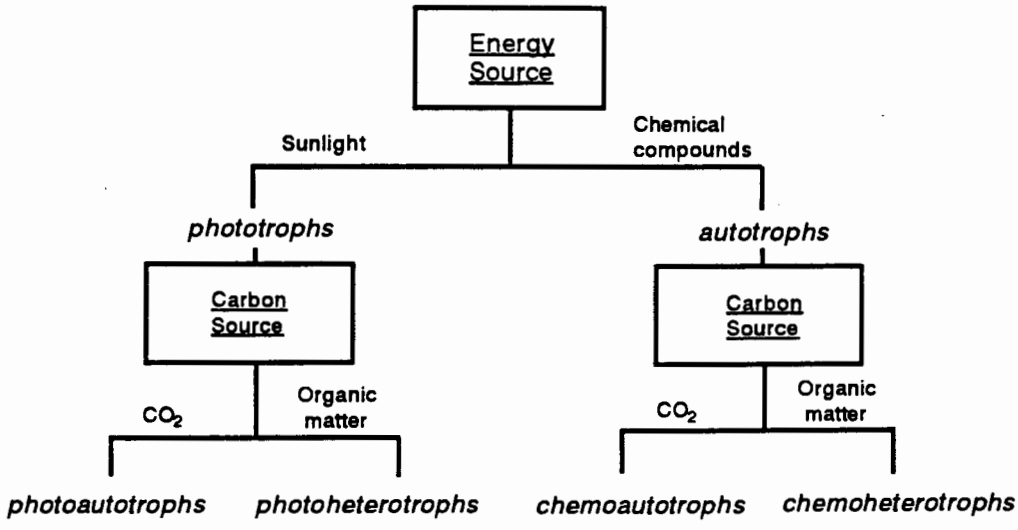

Fig 4.1: Classification of organisms based on their sources of energy and carbon.

respiration. The process in which organisms use the ionic nitrogen oxides, nitrate and/or nitrite as terminal electron acceptor is called *nitrate* or *nitrite respiration.* Organisms which have the ability to switch between oxygen and nitrate/nitrite as electron acceptors in response to their availability are called *facultative* aerobic organisms and are the group of organisms with which this review is primarily concerned. It is clear that such organisms have highly specialized biochemical pathways and the remainder of this review attempts to elucidate the biochemical mechanisms exhibited by heterotrophic facultative organisms under aerobic and anoxic conditions and in changes between the two conditions.

4.3 STAGES OF RESPIRATORY METABOLISM

In the processes of respiratory metabolism by heterotrophic organisms, organic substrates such as carbohydrate, protein and lipids are oxidized to the end products of CO_2 and H_2O. Through these reactions the organism is able to "capture" energy for utilization. Energy becomes available to the organism through a series of internally mediated redox reactions involving electron and proton transfer from a substrate through a number of intermediate enzyme complexes to the final electron acceptor. To capture the energy from these redox reactions, two types of compounds are coupled into the reactions; energy transport molecules which couple, transport, and release energy, and electron and proton transport molecules. The energy transport molecule of interest to this review is *adenosine triphosphate* (ATP). The mechanisms by which ATP participates in energy capture are described at a later stage. The energy transporting molecule, *guanosine triphosphate* (GTP) is also involved in energy capture but is not of major importance to this review. The electron and proton transport molecules of interest are *nicotinamide adenine dinucleotide* (NADH) and *flavin adenine dinucleotide* ($FADH_2$). The NAD molecule belongs to a family of carriers, known as pyridine nucleotides. The oxidized form of NAD, (i.e. NAD^+), which carries a positive charge on the nitrogen atom[2] is

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nitrate concentrations in such systems. Consequently, little attention has been given to the role of nitrite in activated sludge behaviour or kinetics and the term *anoxic* has been considered sufficient for defining situations in which oxygen is absent and nitrate and/or nitrite is present. With increasing attention being devoted to the role of nitrite in wastewater treatment, the present sanitary engineering terminology may have to be reviewed. Although the majority of work reviewed in this Chapter originates from microbiological sources, the major part of this thesis concerns experimental work conducted in the field of sanitary engineering. As a consequence, the terminology applicable to sanitary engineering is used throughout the thesis, including description of the work gained from a literature search in the bacteriological disciplines.

[2]The positive sign associated with the oxidixed form of NAD (NAD^+) is included to

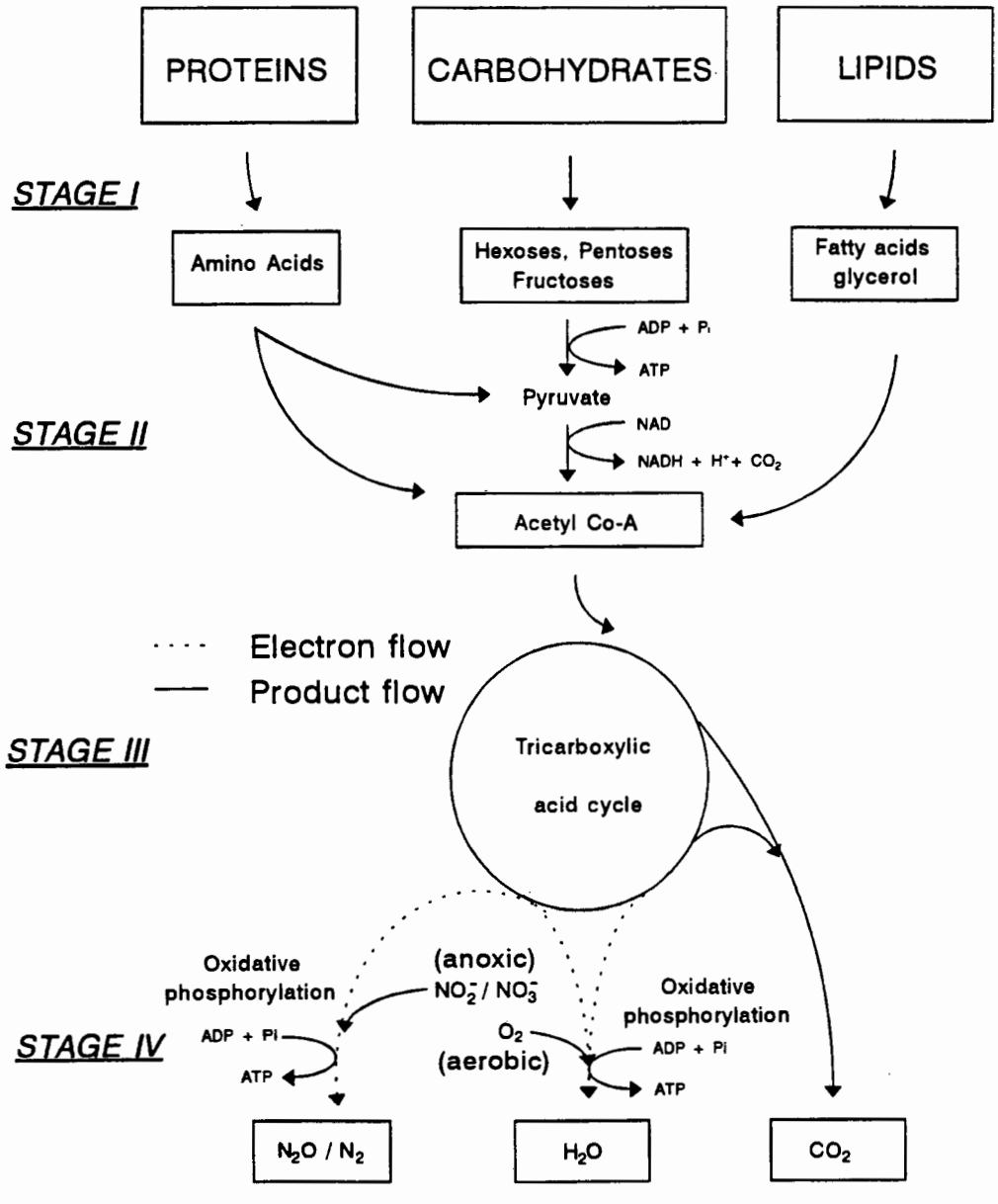

Fig 4.2: Stagewise division of substrate breakdown by facultative heterotrophic organisms, indicating the major metabolic pathways, reactants, and products (from Lehninger, 1975).

formation of pyruvate (from glycine), or without the formation of pyruvate (from tyrosine). Figure 4.4 shows an example of each type of reaction; the degradation of glycine to acetyl–CoA via pyruvate (Fig 4.4a) and the degradation of tyrosine to acetyl–CoA without the formation of pyruvate (Fig 4.4b). Additionally, some amino acids (e.g. glutamate) are not converted to acetyl–CoA but enter Stage III directly.

Carbohydrate degradation (glycolysis) occurs via a number of pathways for facultative organisms the most common of which is the Embden–Meyerhof pathway. Essentially it can be considered as a 2–stage (10 step) process as illustrated in Fig 4.5. The first stage is an activation stage in which simple sugars (e.g. glucose, fructose) are phosphorylated to a common product, glyceraldehyde 3–phosphate at the expense of ATP. The second stage comprises a series of dehydrogenations (i.e. removal of protons and electrons) by which glyceraldehyde 3–phosphate is converted to pyruvate. Associated with this stage is the formation of NADH and ATP, the formation of the latter a consequence of oxidative phosphorylation.

Hydrolysis of long chain fatty acids occurs by a process termed β–oxidation in which acetyl–CoA, NADH and $FADH_2$ are formed at the expense of ATP via one hydration and two dehydrogenation steps as illustrated in Fig 4.6.

In summary, although many products are generated by the degradation of carbohydrates, proteins and lipids through Stages I and II, those which are of principal interest to this review are, acetyl–CoA, ATP, NADH and $FADH_2$.

Stage III

The end product from Stage II, acetyl–CoA, enters the tricarboxylic acid (TCA) cycle, a cyclic sequence of reactions catalyzed by a series of enzymes. During the cycle the acetyl group of acetyl–CoA is oxidized to form two molecules of CO_2, eight protons (H^+), 4 pairs of electrons (e^-) [3] *and 1 GTP (energetically equivalent to 1 ATP); CoenzymeA is recovered. The TCA cycle (see Fig 4.7), also known as the Krebs or citric acid cycle, is the final catabolic pathway common to all aerobic and facultative organisms.*

Six of the protons and 3 pairs of electrons produced in the cycle are transferred to 3

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[3]The terminology "pairs of electrons" is used to indicate that electrons (e^-) are transported as pairs, unlike protons (H^+) which can be transported individually.

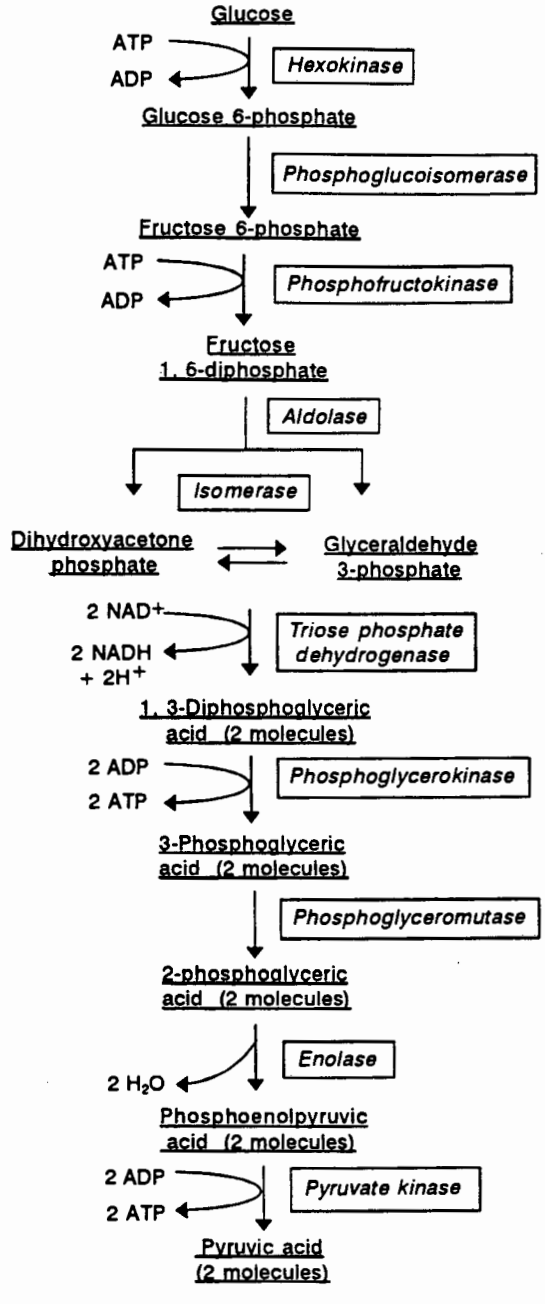

Fig 4.5: Ten step degradation of carbohydrate (glycolysis), demonstrating collection of simple sugars and production of glyceraldehyde 3–phosphate, and conversion of glyceraldehyde 3–phosphate to pyruvic acid with coupled formation of ATP (redrawn from Lehninger, 1975).

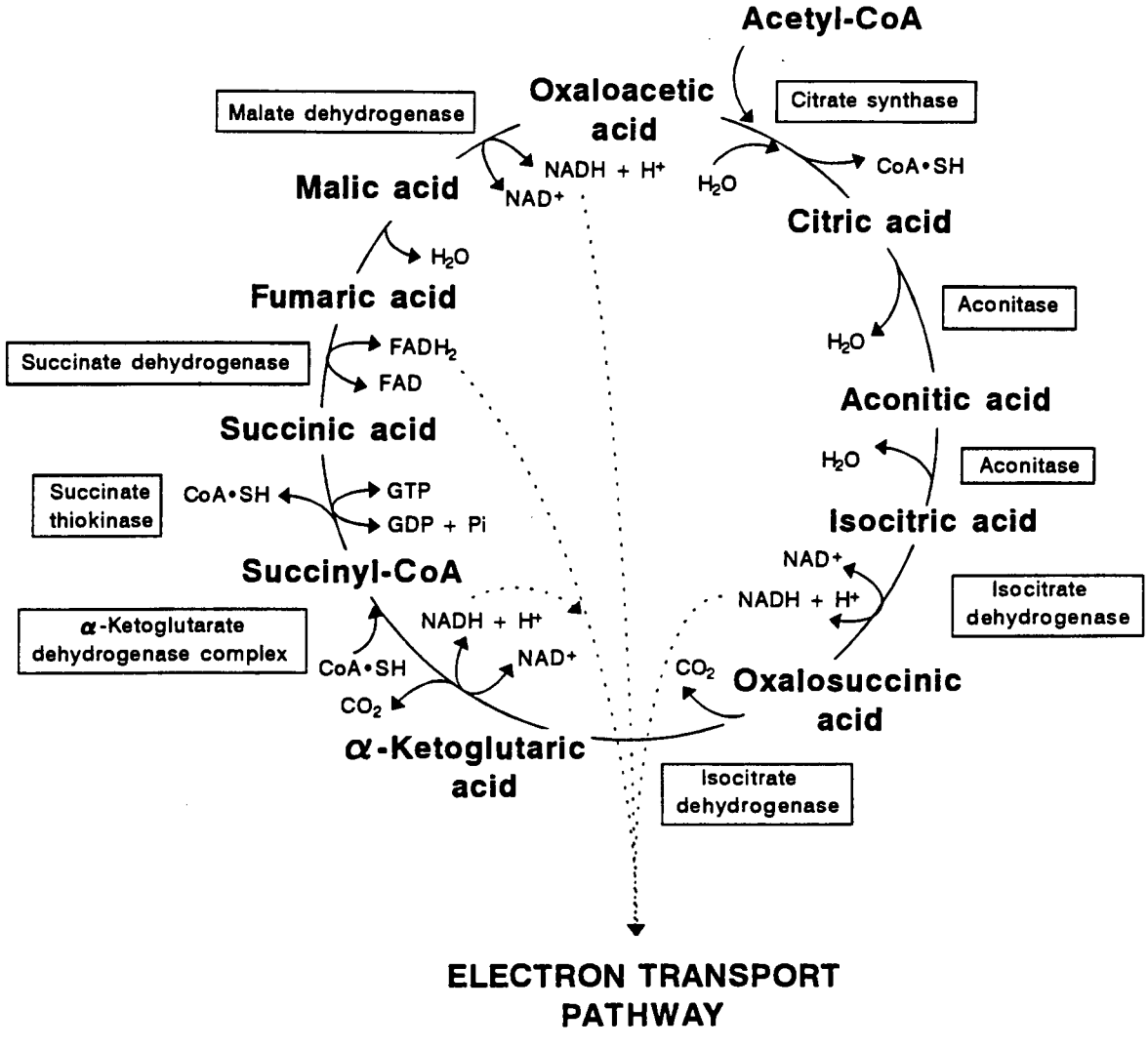

Fig 4.7: Tricarboxylic acid (Krebs) cycle demonstrating the formation of NADH and $FADH_2$ with reference to acetyl–CoA and the electron transport pathway.

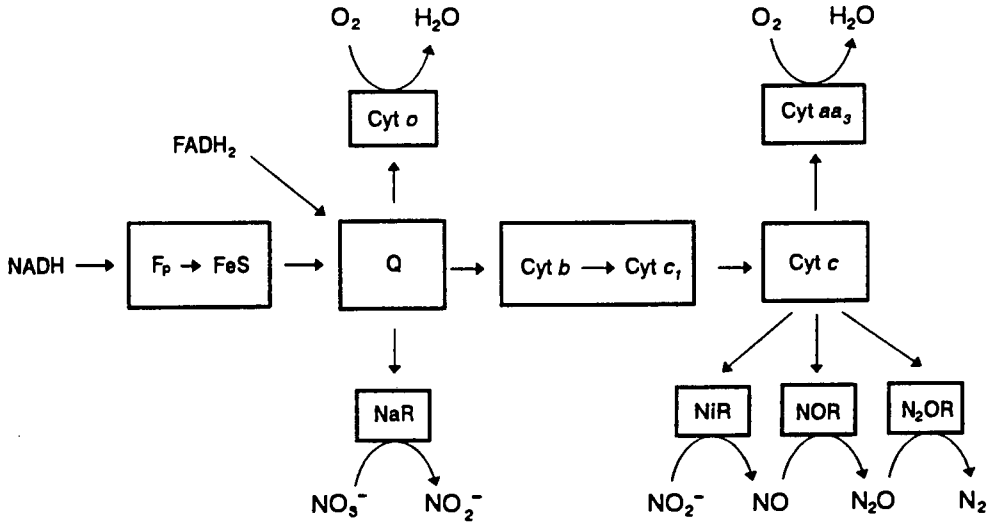

Fig 4.8: Electron transport pathway (ETP) for a typical facultative aerobic heterotrophic organism indicating the points of transfer of electrons to the terminal oxidases and reductases. [Abbreviations: NADH – nicotinamide adenine dinucleotide; Fp – flavoprotein; FeS – iron sulphur complexes; FAD – flavin adenine dinucleotide; Q – ubiquinone, Cyt – Cytochrome; NaR – nitrate reductase; NiR – nitrite reductase; NOR – nitric oxide reductase; N_2OR – nitrous oxide reductase; ADP – adenosine diphosphate; ATP – adenosine triphosphate]. (Adapted from Ferguson, 1982).

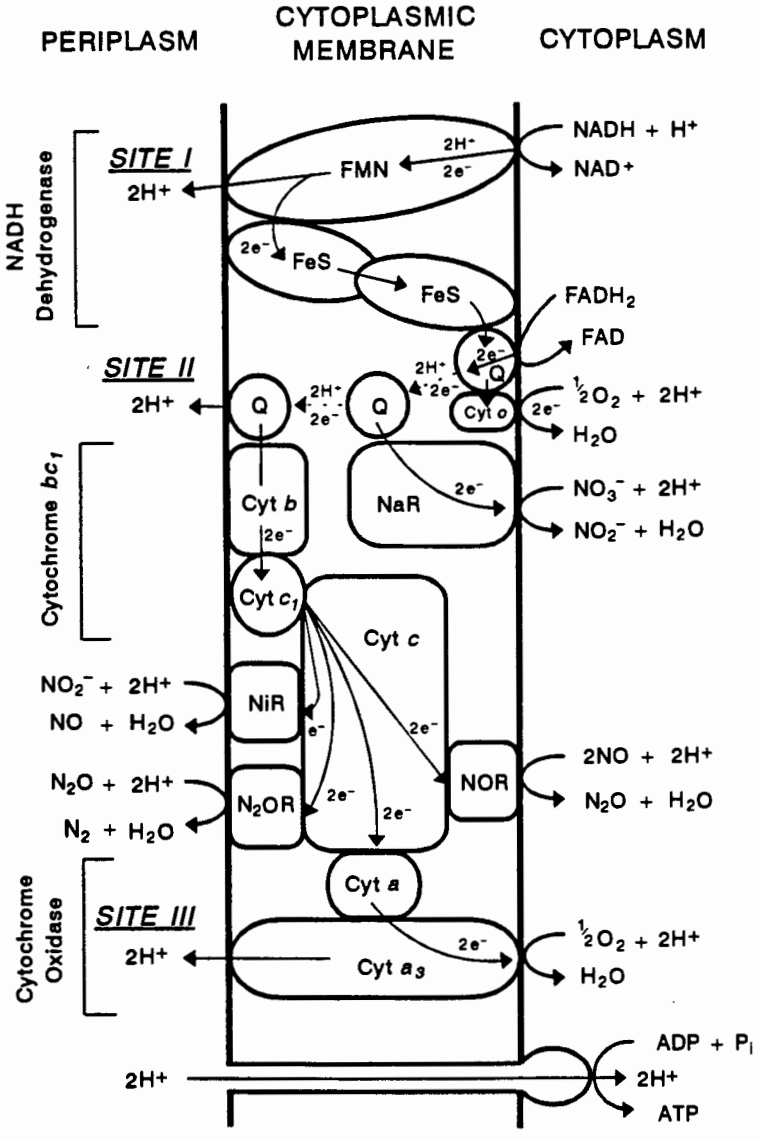

Fig 4.9: Electron transport pathway (ETP) for a typical facultative aerobic heterotrophic organism indicating the active sites of both the aerobic and anoxic electron transferring complexes with respect to the cytoplasmic membrane and the three energy conserving (proton-pumping) sites. [Abbreviations: NAD – nicotinamide adenine dinucleotide; FMN – flavomononucleotide; FeS – iron sulphur complexes; FAD – flavin adenine dinucleotide; Q – ubiquinone, Cyt – Cytochrome; NaR – nitrate reductase; NiR – nitrite reductase; NOR – nitric oxide reductase; N_2OR – nitrous oxide reductase; ADP – adenosine diphosphate; ATP – adenosine triphosphate].

mononucleotide (FMN) or *flavin* which acts as the electron and proton transferring site of the polypeptide.

The NADH dehydrogenase complex catalyzes the transfer of two protons and a pair of electrons from NADH to ubiquinone in three steps.

In the first step, the prosthetic group, FMN of the flavoprotein (Fp) (which spans, and is exposed on both sides of the membrane), gains two protons and a pair of electrons to give the reduced form $FMNH_2$. One proton and a pair of electrons are donated from NADH on the cytoplasmic side of the membrane and the other proton, (the one left free in the formation of NADH), is gained from the cytoplasmic medium. The overall transfer of protons and electrons from NADH to FMN is as follows:

$$NADH + H^+ + FMN \rightarrow FMNH_2 + NAD^+ \qquad (4.3)$$

In the second step, a pair of electrons are transferred from $FMNH_2$ to a series of iron–sulphur protein complexes (FeS) on the periplasmic side of the membrane at which point the two protons are released to the periplasm. The net result is the transfer of two protons from the cytoplasmic side of the membrane to the periplasmic side. The point at which protons are extruded through the membrane to the periplasm is conventionally regarded as the first of three proton–pumping or energy conserving positions along the pathway and is referred to as *Site I* (see Section 4.6 below for a full description of the mechanism of energy formation). In accepting a pair of electrons from $FMNH_2$, each of the iron atom(s) of the FeS complexes undergoes a valency change[5], from the oxidized ferric (+3) state to the reduced ferrous (+2) state as follows:

$$FMNH_2 + 2Fe^{3+} \rightarrow 2Fe^{2+} + FMN + 2H^+ \qquad (4.4)$$

In the third step, a pair of electrons are transferred between the FeS complexes, and each reduced FeS complex donates one electron to the next carrier in the ETC, ubiquinone.

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[5]Although the FeS complexes are catalytic in that they switch between the oxidized and reduced states, the reaction is not shown as a reversible reduction–oxidation reaction because Fe^{2+} reverts to the Fe^{3+} form only following transfer of electrons to the next electron transferring molecule, ubiquinone.

An important characteristic of cytochromes is that they are able to transfer electrons only, unlike ubiquinone, NADH, NADH dehydrogenase, $FADH_2$ and ubiquinone which transport both electrons and protons.

Cytochrome bc_1 complex

The cytochrome bc_1 complex contains two sequential electron transporting proteins, cytochrome *b* and cytochrome c_1. The role of the cytochromes of the cytochrome bc_1 complex is to act as an intermediate in the transfer of electrons from ubiquinol to cytochrome *c*, the next complex in the pathway. In the cytochrome bc_1 complex, a pair of electrons are transferred sequentially from ubiquinol to cytochrome *b*, then to cytochrome c_1, and then to the next electron transporting complex, cytochrome *c*. Cytochromes are electron transporting proteins which belong to a class of haem (iron–containing) proteins which also includes haemoglobin. All proteins in the group contain haem in a prosthetic (attached) group. Cytochrome *b*, cytochrome c_1 and cytochrome *c* all contain the same haem group, the difference between the three being the type of bond by which the group is attached to the protein. In cytochrome *b* the haem is not covalently bonded to the protein whereas in cytochrome *c* and cytochrome c_1 the haem is attached to the protein by covalent bonds. As with the iron atom of the FeS complexes, the iron atom in the haem group of the cytochromes alternates between an oxidized ferric (+3) state and a reduced ferrous (+2) state during electron transport. Regarding electron transport, it should be noted that the haem groups of the cytochromes are able to transfer only one electron at a time, unlike NADH, $FMNH_2$ and ubiquinone which transfer two electrons. Thus even though it is not shown as such in Fig 4.10, for each molecule of ubiquinol carrying two electrons and two protons, two molecules of cytochrome *b*, cytochrome c_1, and cytochrome *c* are required.

Cytochrome *c* complex

The cytochrome *c* complex receives electrons from cytochrome c_1 of the cytochrome bc_1 complex and transfers them to the terminal electron transferring complex for aerobic respiration, the cytochrome oxidase complex, cytochrome aa_3.

Cytochrome oxidase complexes

The terminal complex in the aerobic electron transport pathway, the cytochrome oxidase complex, transfers electrons to the terminal electron acceptor, oxygen. For obligate aerobic organisms, the aerobic cytochromes in the electron transport pathway to oxygen are contained in the cytochrome aa_3 complex. For

Under the environmental conditions in which cytochrome *o* acts as oxidase, electrons flow directly from ubiquinone to cytochrome *o*; cytochromes bc_1 and *c* are not involved. Accordingly, the electrons pass only 2 energy conserving sites, Sites I and II, for NADH as electron donor and one site, Site II, for $FADH_2$ as electron donor. For NADH as electron donor, electrons are transferred to NADH dehydrogenase, then to ubiquinone and for $FADH_2$ as electron donor, to ubiquinone directly. For cytochrome *o* as oxidase, 4 protons from the cytoplasm combine with an oxygen molecule and 2 pairs of electrons from ubiquinone to form water as follows:

$$4\text{cyt } o\ (Fe^{2+}) + O_2 + 4H^+ \rightarrow 2H_2O + 4\text{cyt } o\ (Fe^{3+}) \qquad (4.9)$$

The environmental conditions under which cytochrome *o* or cytochrome aa_3 act as terminal electron acceptors are described in Part II of this review.

A significant physical difference between cytochrome *o* and cytochrome aa_3 is in the metal electron transferring centres contained in each cytochrome. Cytochrome *o* contains iron–sulphur (FeS) complexes, whereas cytochrome aa_3 contains iron–sulphur and copper–sulphur (FeS and CuS) centres. The implications of this difference will become apparent in Part II of this review – in investigation of the mechanisms of regulation of respiration. The active site of cytochrome *o* has been assigned to the cytoplasmic side of the membrane since measurements of electron–transport–linked proton translocation indicate that protons required for reduction of O_2 are taken from the cytoplasm, (Boogerd *et al.*, 1981; Willison and Haddock, 1981). Van Verseveld *et al.* (1981) proposed also a cytoplasmic orientation for cytochrome a_3.

4.5 ANOXIC RESPIRATION

The description above of aerobic respiration is for conditions where dissolved oxygen (DO) is in adequate supply. When DO becomes limiting, facultative heterotrophs are able to switch from oxygen to nitrate/nitrite as terminal electron acceptor and respire anoxically. For nitrate/nitrite as electron acceptor, the first three stages and the greater part of the fourth stage of the electron transport pathway continue functioning unchanged; as mentioned in passing above, a difference in electron transport between aerobic and anoxic respiration becomes apparent only after the ubiquinone complex, i.e. in the electron and proton transferring complexes of cytochrome steps in Stage IV of Fig 4.2. Under anoxic conditions not all of the

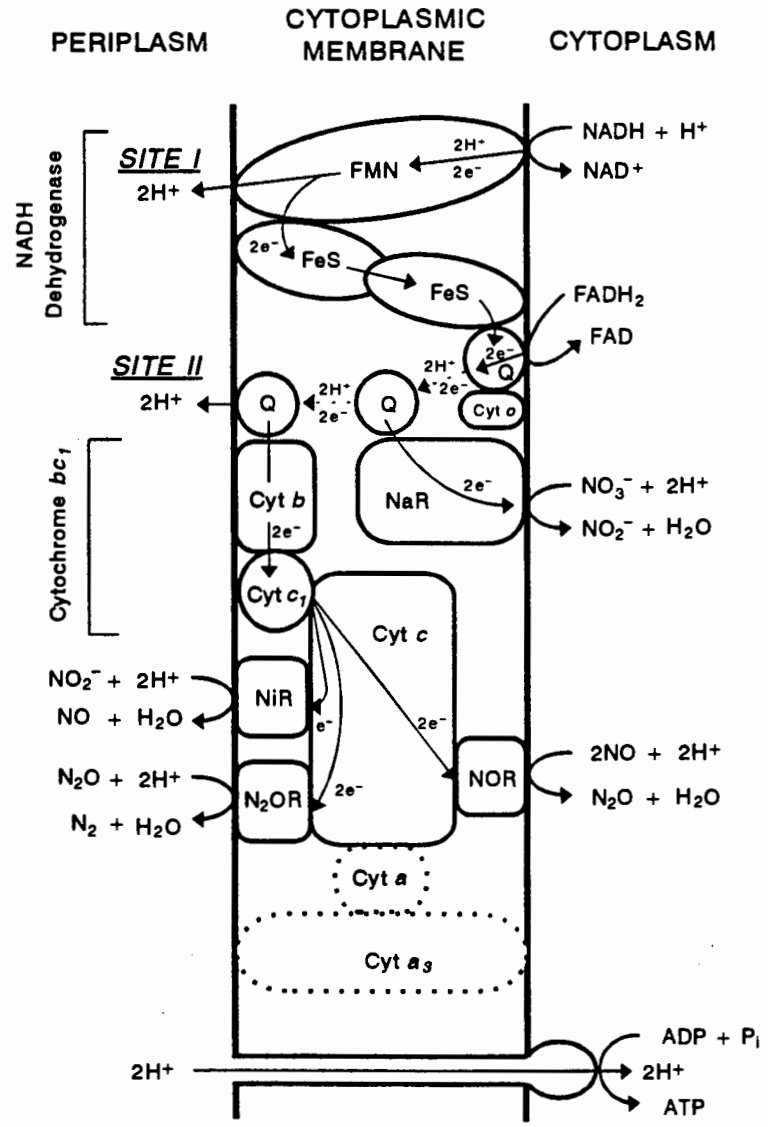

Fig 4.11: Electron transport pathway (ETP) for a typical facultative aerobic organism grown under anoxic conditions, indicating the presence of the reductases and cytochrome *o*, and the synthesis at a low level of the oxidase, cytochrome aa_3. [Abbreviations: NAD – nicotinamide adenine dinucleotide; FMN – flavomononucleotide; FeS – iron sulphur complexes; FAD – flavin adenine dinucleotide; Q – ubiquinone, Cyt – Cytochrome; NaR – nitrate reductase; NiR – nitrite reductase; NOR – nitric oxide reductase; N_2OR – nitrous oxide reductase; ADP – adenosine diphosphate; ATP – adenosine triphosphate].

ubiquinone via the γ–subunit to the iron–sulphur and molybdenum centres of the α–subunit, none of which can occur without the association of the β and γ–subunits with the membrane as illustrated in Fig 4.12. From the catalytic site at the α–subunit, 2 electrons are passed to nitrate which in the process also takes up 2 protons from the periplasm to form nitrite and water as follows:

$$2H^+ + 2e^- + NO_3^- \rightarrow NO_2^- + H_2O \qquad (4.11)$$

In the process of transferring 2 electrons to nitrate reductase, ubiquinone ejects 2 protons to the periplasm and establishes the site of NO_3^- reduction as the second (Site II) of the energy conserving (proton–pumping) sites of the anoxic respiratory pathway as indicated in Fig 4.11. This site corresponds to Site II of aerobic respiration at which ubiquinone ejects 2 protons to the periplasm in the process of transferring electrons to the cytochrome bc_1 complex. Thus, for each molecule of nitrate reduced, 2 electrons originating from NADH pass 2 proton–pumping sites, Sites I and II, at each of which 2 protons are translocated to the periplasm. Electrons originating from $FADH_2$ and transferred to nitrate pass only one proton–pumping site, Site II.

Given the cytoplasmic placement of the active site of nitrate reductase, nitrate has to be translocated from outside to inside the cytoplasmic membrane. The literature is not clear on the precise mechanism for this translocation. John (1977) proposed a transmembrane ionic carrier specific to nitrate. Boogerd *et al.* (1983a) proposed two uptake systems for nitrate which operate in sequence. The first system is an H^+–NO_3^- symport mechanism which initiates nitrate uptake and is dependent on the proton motive force (pmf) established by the translocation of protons across the cytoplasmic membrane. Nitrate crosses the membrane together with two or more protons during production of ATP, and is reduced to nitrite at the α–subunit on the cytoplasmic side. The second nitrate uptake system is an NO_3^-/NO_2^- antiport, the function of which is to take over NO_3^- uptake from the first system. For each nitrate molecule crossing the membrane to the cytoplasm, one nitrite molecule passes back to the periplasm. The symport and antiport mechanisms are illustrated in Fig 4.13. However, because no physical evidence exists for the presence of such systems, Craske and Ferguson (1986) suggested that the nitrate reductase complex itself incorporates a nitrate–specific channel which provides access for nitrate to the active site of its reductase.

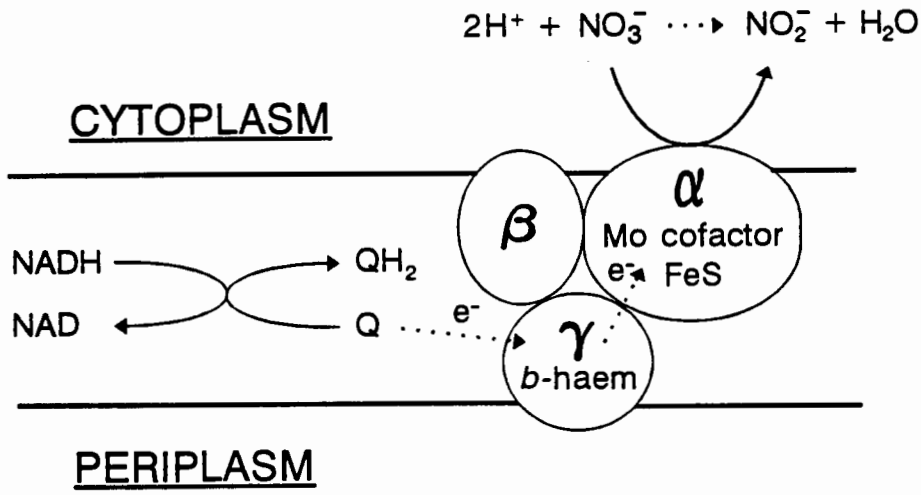

Fig 4.12: Subunits of nitrate reductase indicating the position of each with respect to the membrane and the role of each in electron transport to nitrate. [Abbreviations: NAD – nicotinamide adenine dinucleotide; Q – ubiquinone; Mo – molybdenum; FeS – iron–sulphur]. (redrawn from Stewart, 1988)

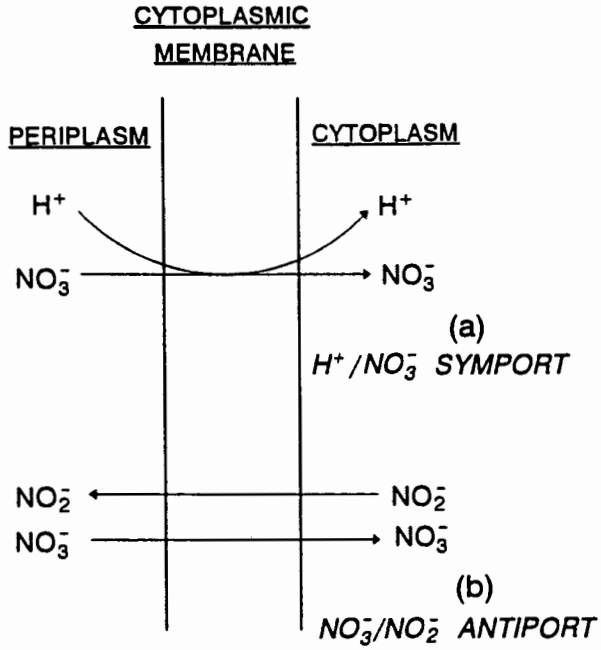

Fig 4.13: Illustration of the mechanism of (a) an H^+/NO_3^- symport and, (b) an NO_3^-/NO_2^- antiport nitrate transport systems (redrawn from Stouthamer, 1988).

Nitric Oxide Reductase

The function of nitric oxide reductase is to reduce nitric oxide to nitrous oxide. Nitric oxide reductase, situated on the cytoplasmic side of the membrane, (Heiss *et al.*, 1989, Carr *et al.*, 1989) receives 2 electrons from cytochrome *c* and at the catalytic site of the reductase the electrons are passed to two molecules of nitric oxide which in the process takes up 2 protons from the cytoplasm to form one molecule of nitrous oxide and water as follows:

$$2H^+ + 2e^- + 2NO \rightarrow N_2O + H_2O \qquad (4.13)$$

The two electrons gained by nitric oxide were transferred between the same complexes as electrons gained by nitrite, i.e. electrons passed the same two proton–pumping sites, Sites I and II, for electrons originating from NADH and Site II only for electrons originating from $FADH_2$. Thus the same number of protons (4) are pumped across the membrane per 2 electrons gained by nitric oxide as for nitrite, which equals the number of protons pumped across the membrane per 2 electrons gained by nitrate.

Transfer of electrons from nitric oxide reductase to nitric oxide occurs via the reactive centre of the reductase, a *bc*–type haem. The cytoplasmic placement of nitric oxide reductase necessitates the movement of nitric oxide across the membrane to the reductase and the movement of the product nitrous oxide reductase from the cytoplasm to the periplasm, where it is either reduced by its reductase or excreted extracellularly. However no active or passive transport system has yet been found for these molecules. An interesting aspect concerning nitric oxide reductase is that in the formation of nitrous oxide from nitric oxide a dinitrogen bond is formed, which is necessary for the ultimate production of dinitrogen (N_2).

Nitrous oxide reductase

The function of nitrous oxide reductase is to reduce N_2O to N_2. Nitrous oxide reductase situated on the periplasmic side of the membrane (Boogerd *et al.*, 1981) receives 2 electrons from cytochrome *c* and at the catalytic site of the reductase the electrons are passed to nitrous oxide which in the process takes up 2 protons from the periplasm as follows:

$$2H^+ + 2e^- + N_2O \rightarrow N_2 + H_2O \qquad (4.14)$$

Site I is at the NADH dehydrogenase complex, where the flavoprotein (Fp) complex expels 2 protons to the periplasm for each pair of electrons transferred to the next electron transferring complex, the iron–sulphur (FeS) complex. Site II is at the point at which the ubiquinone (Q) molecule expels 2 protons to the periplasm during the transfer of a pair of electrons to the cytochrome bc_1 complex. Site III is at the oxidase complex cytochrome aa_3, at which point 2 protons are expelled to the periplasm and 2 electrons are transferred to oxygen during its reduction to water. This coupling of respiration and oxidative phosphorylation was proposed in 1961 by Mitchell and is known as the chemiosmotic theory.

The first step in oxidative phosphorylation involves the translocation of protons across the normally proton–impermeable cytoplasmic membrane during the passage of electrons through the electron transport pathway in the manner described above. The mechanism by which this movement of protons is coupled to energy production is illustrated in Fig 4.14 and can be described as follows: the translocation of protons from the cytoplasmic to the periplasmic side of the membrane establishes an electrochemical potential across the membrane referred to as proton motive force (pmf). Proton motive force ($\Delta\mu_H$) has both a pH and a charge ($\Delta\psi$) component which can be expressed as follows:

$$\Delta\mu_H = \Delta\psi + \frac{2{,}3RT}{F}\Delta pH \qquad (4.15)$$

where

R = Universal gas constant (1,987 cal/mol.K)

T = Temperature (K)

F = Faraday's constant (23062 cal/volt)

Proton motive force is a form of potential energy and as the name implies acts to drive the protons on the periplasmic side of the membrane back to the cytoplasmic side during which process, energy is converted from a potential form to a chemical form. The protons pass through the membrane from the periplasm to the cytoplasm via a channel created by a protein called adenosine triphosphatase (ATPase). This complex molecule spans the cytoplasmic membrane and is composed of three protein units denoted F_0, F_1 and I. The F_0 protein transfers protons across the lipid layer of the membrane; the F_1 protein catalyzes the phosphorylation of ADP to ATP; and the I protein is a regulatory protein that prevents premature hydrolysis of the newly formed ATP. The energy–rich ATP serves as an energy source for the

organism. To generate one molecule of ATP, two protons must transverse the membrane via the ATPase enzyme. Accordingly, one ATP will be generated for each pair of protons pumped across the membrane at each of Sites I, II and III, or in other words, for aerobic respiration with a pair of electrons flowing past Sites I to III, 3 ATPs will be generated per electron pair passing from $NADH_2$ to O_2, and for anoxic respiration with a pair of electrons flowing past Sites I and II only, 2 ATPs will be generated per electron pair passing from $NADH_2$ to one of the nitrogen oxides.

Establishing the position of the energy sites in the electron transport pathway

The chemiosmotic theory hinges on the certain identification of the position and function of the three proton–pumping sites described above. These sites have been identified experimentally through three different approaches; (i) comparison of the ATP yield of different substrates, (ii) thermodynamic estimates of electron and proton flow between complexes, and (iii) inhibition of electron flow at specific sites on the electron transport pathway (Stryer, 1981).

In the first approach, artificial substrates are used which donate electrons and protons at different positions on the electron transport pathway producing different growth yields. For example, it is known that under aerobic conditions the electron and proton donors, NADH, succinate and ascorbate, donate electrons to NADH dehydrogenase, ubiquinone and cytochrome *c* respectively, as shown in Fig 4.15. Theoretically, electrons supplied by NADH pass Sites I, II, and III, yielding 3 moles of ATP per mole of electrons passed to oxygen; electrons supplied by succinate pass Sites II and III only, yielding 2 moles of ATP per mole of electrons passed to oxygen and; electrons supplied by ascorbate pass Site III only, yielding 1 mole of ATP per mole of electrons supplied to oxygen. Experiments conducted with NADH, succinate, and ascorbate as substrates produced organism growths in the approximate ratio, 3:2:1 respectively, providing support for the identification of the 3 sites at the complexes described above (Stryer, 1981).

In the second approach, from the known reduction–oxidation (redox) potentials for each cytochrome couple on the pathway, the free energy gained through transmission of electrons between cytochromes can be calculated and compared to the known free energy required to drive the synthesis of ATP. The relationship between the redox potentials and corresponding free energies for each cytochrome complex are shown diagrammatically in Fig 4.16. At each of Sites I, II and III the

change in redox potential is such that sufficient free energy is released for the synthesis of ATP. The standard free energy ($\Delta G_o'$) generated by the transfer of electrons between two complexes can be calculated from the standard reduction–oxidation (redox) potentials of the complexes:

$$\Delta G_o' = -n\ F\Delta E_h' \qquad (4.16)$$

where

n = number of electrons transferred in the reaction

F = Faraday constant (23 062 cal/volt)

E_h' = redox potential difference between complexes (mV)

The third approach involves the use of known electron transport inhibitors which block electron transport at specific points on the ETP. In this way, electrons pass 1, 2, or 3 proton–pumping sites depending on the inhibitor used. For example, the inhibitors rotenone and amytal prevent electron flow proceeding past the NADH dehydrogenase complex, antimycin A prevents electron flow proceeding past cytochrome *b*, and cyanide and carbon monoxide prevent electron flow to cytochrome a_3. Figure 4.15 indicates the points on the ETP at which each inhibitor and other inhibitors act. The observed growth yields with the use of these chemicals supported the proposed positions of the three proton–pumping sites. The details of these experiments are described in Appendices C and D.

These three approaches established the position of the three proton–pumping – energy conserving sites and are useful tools in determining the efficiency of growth under aerobic and anoxic conditions in which oxygen and nitrogen oxides respectively serve as terminal electron acceptors at different positions on the electron transport pathway. The necessity for establishing the positions on the aerobic electron transport pathway of the three proton–pumping sites will become evident in the following section which investigates the relationship between the electron transferring pathways involved in aerobic and anoxic respiration.

<u>Energetic yield associated with aerobic and anoxic growth</u>

Considering Fig 4.9 as typical of the cytochromes present and reactions associated with a facultative organism, it is possible to determine the comparative ATP yields associated with the use of oxygen and the nitrogen oxides as terminal electron acceptors.

Considering the first case: For denitrifying organisms the reduction of one nitrate ion through each nitrogen oxide intermediate to a dinitrogen molecule requires four pairs of electrons. During transfer through the denitrification complexes to the nitrogen oxides, the electrons pass two energy conserving sites. Sites I and II (i.e. one pair of electrons is required for the reduction of each nitrogen oxide intermediate to the next intermediate), resulting in the transferal of 4 pairs of protons across the membrane and the concomitant generation of 4 ATP. In comparison, for nitrate reducing organisms, the reduction of the nitrate ion to a nitrite ion requires only one pair of electrons, which during transfer from NADH through the denitrification complexes passes the first two energy conserving sites, resulting in the generation of 2 ATP, a considerably lower energetic yield than that gained by the denitrifying organisms.

Considering the second case: For denitrifying organisms, the reduction of a nitrite ion through each nitrogen oxide intermediate to a dinitrogen molecule requires three pairs of electrons, which during transfer from NADH through the denitrification complexes to one of the nitrogen oxides, pass Sites I and II, resulting in the generation of 3 ATP. In comparison, nitrate reducers cannot utilize nitrite and gain no energy from its presence under anoxic conditions.

PART II: MECHANISMS OF REGULATION OF AEROBIC AND ANOXIC RESPIRATION IN FACULTATIVE ORGANISMS

4.7 INTRODUCTION

In Part I the biochemical pathways and enzyme complexes present in facultative organisms under steady–state aerobic and steady–state anoxic conditions were described. Also, it was established that the enzyme complexes (the oxidases and nitrogen oxide reductases) which transfer electrons to the terminal electron acceptors are adaptive, i.e. are synthesized when aerobic or anoxic conditions respectively are imposed. Facultative organisms have the propensity to respire under anoxic or aerobic conditions. In nitrogen and nutrient removal activated sludge systems, these organisms are constantly cycled between anoxic and aerobic conditions. This raises the question as to how the organisms regulate the synthesis and activity of the required enzyme complexes when changing from anoxic to aerobic conditions and *vice versa*.

In this section – Part II – it is the intention to examine the factors associated with initiation, regulation, and termination of aerobic and anoxic respiration. Each of

conditions and total absence when grown under anoxic conditions, and although cytochrome *o* was synthesized under both aerobic and anoxic/anaerobic conditions, under aerobic conditions it developed to only 70% of its level under anoxic conditions.

Under oxygen saturation conditions, synthesis of nitrate reductase of the nitrate reducing organism *E. coli* is reduced to its basal level (Showe and de Moss, 1968) and similarly for the denitrifying organism *Pa. denitrificans* nitrate and nitrite reductase were measured at a low level (Lam and Nicholas, 1969a).

Requirements for induction of denitrifying enzyme synthesis

When aerobic conditions are changed to anoxic conditions, the potential exists for induction of synthesis of the denitrifying enzymes and concomitant denitrification. (Although nitrate reductase and the other nitrogen oxide reductases are present under aerobic conditions at low levels, with the onset of anoxic conditions, the level of enzyme synthesis is too low to allow an immediate measurable rate of denitrification). Considerable variability exists between organisms in their requirements for synthesis of the denitrifying enzymes. Most organisms, e.g. *Pa. denitrificans, Klebsiella aerogenes* and *Alcaligenes faecalis* require the absence of oxygen for derepression of nitrate and nitrite reductase and the presence of nitrate and nitrite for induction of nitrate and nitrite reductase synthesis respectively (Lam and Nicholas, 1969a; Pichinoty and d'Ornano, 1961c; Kakutani *et al.*, 1981). Other organisms such as *Bacillus licheniformis, Proteus mirabilis and Ps. stutzeri* require only the absence of oxygen for nitrate and nitrite reductase synthesis (Schulp and Stouthamer, 1970; de Groot and Stouthamer, 1970b; Kodama, 1970), but even in these organisms nitrate and nitrite reductase is synthesized at only about 40 per cent of the level to which it is synthesized in the presence of nitrate and nitrite. Under aerobic conditions, synthesis of the reductases is *repressed*, and nitrous oxide more so than the others; in aerobically grown *Ps. perfectomarinus* switched to anoxic conditions in the absence of nitrate and nitrite, synthesis of nitrous oxide reductase began first (Payne, 1973). For aerobically grown *Pa. denitrificans* switched to anoxic conditions, synthesis of all the denitrifying enzymes began simultaneously (Payne, 1973).

To examine the relative importance of oxygen and nitrate concentration on the synthesis of nitrate reductase, *Pa. denitrificans* was grown at different concentrations of oxygen with nitrate present (Sapshead and Wimpenny, 1972). At

Control of the *synthesis* of the reductases is exerted at the level of expression of the specific gene for synthesis of the reductases. The details of this mechanism are given in Appendix D.

Control of the *activity* of the denitrifying enzymes under anoxic conditions involves complex interactions between the nitrogen oxides and the nitrogen oxide reductases. Each of the nitrogen oxides interacts with its own reductase and other reductases, the nett effect of which is a mechanism which ensures that none of the nitrogen oxide intermediates, (NO_2^-, NO, N_2O) increases to high concentrations. A summary of the results of experimental work conducted on the interactions between the nitrogen oxides and the nitrogen oxide reductases is given in Table 4.1. The interactions in which each intermediate inactivates each nitrogen oxide reductase other than its own appears to be virtually general, except for interactions of nitric oxide with nitrate reductase and nitrate with nitrous oxide reductase. In the literature search, no report appears to have yet been published on these interactions. Additionally the interaction of nitrate with nitrite reductase appears to be anomalous in that it is not repressive, but is an activating interaction. This effect has been confirmed in four out of five investigations. An explanation is that the end-product of nitrate reduction, i.e. nitrite, activates nitrite reductase.

Effect of aerobic growth conditions on the synthesis of the denitrifying enzymes of organisms isolated from activated sludge

In the foregoing section, the effects of aerobic and anoxic conditions on the aerobic and denitrifying enzymes of organisms in pure culture have been examined. Because of the applied nature of this investigation it would be advantageous to establish whether similar behaviour occurs in facultative organisms from activated sludge. In this section the effects of similar conditions on the denitrifying enzymes of facultative organisms from activated sludge are reviewed.

In 24 facultative bacteria isolated from denitrifying and aerobic sludges, the synthesis of nitrate reductase after growth under aerobic conditions varied from total repression in some bacteria to near non-repression in others (Krul and Veeningen, 1977).

In a similar investigation (Simpkin and Boyle, 1988), the level of synthesis of nitrate and nitrite reductase was analysed for a mixed culture activated sludge subjected to various aerobic/anoxic conditions. The synthesis of these reductases was repressed

by less than 50% on exposure to aerobic conditions in comparison to almost complete repression for the pure culture organisms described above. The authors concluded that less than 50% change in synthesis of nitrate and nitrite reductase between aerobic and anoxic growth could not account for the very large difference in nitrate and nitrite reduction between aerobic and anoxic conditions and attributed the difference to the effect of oxygen on the *activity* and not the *synthesis* of the reductases.

4.9 ANOXIC CONDITIONS CHANGED TO AEROBIC CONDITIONS

Inhibition of activity and repression of synthesis of the denitrifying enzymes

It is generally accepted that oxygen has an immediate inhibitory effect on denitrification. It can be assumed that upon exposure to oxygen the immediate reduction in denitrification rate does not result from changes in the level of synthesized enzymes, but from a change in the activity of the denitrification enzymes.

To investigate the effect of oxygen on denitrification generally, facultative organisms were subjected to slowly increasing concentrations of dissolved oxygen. From work conducted with *Hyphomicrobium* X (Meiberg *et al.,* 1980) and *Thiobacillus denitrificans* (Justin and Kelly, 1978) it was established that low concentrations of oxygen inhibit the activity of the nitrogen oxide reductases and higher concentrations repress synthesis of the enzymes. Hochstein *et al.* (1984) demonstrated that for the activities of the reductases of *Pa. halodenitrificans* the order of decreasing sensitivity to oxygen is; nitrous oxide–, nitrite–, and nitrate reductase.[9] Thus, under conditions of increasing concentration of dissolved oxygen, but at oxygen concentrations at which enzyme synthesis is not repressed, the products and intermediates of denitrification appeared in the order; dinitrogen, nitrous oxide, and then nitrite. Tiedje (1985) illustrates the effect of oxygen at different concentrations on the activity and synthesis of the denitrifying reductases of *Hyphomicrobium* in the manner of Fig 4.17. Because the author did not accept nitric oxide as an obligatory intermediate in the denitrification pathway, it was not included in the original work, but is included here between nitrite reductase and

[9]Because of its very rapid instability in aerobic environments, nitric oxide does not appear as an external intermediate during inhibition of nitrogen oxide activity by oxygen. In these experiments nitric oxide was not measured and consequently the workers did not consider nitric oxide as an obligatory compound in the denitrification pathway and thus did not consider the effect of oxygen on nitric oxide reductase.

nitrous oxide reductase.

In order to examine more specifically the effect of oxygen on the activity of denitrifying enzymes, workers grew organisms under anoxic conditions such that the denitrifying enzymes were fully synthesized and then exposed the organisms to aerobic conditions which caused that denitrification was reduced or terminated and closely monitored the electron transferring capacity of the enzymes. From these investigations, the inactivation of the denitrifying enzymes (and nitrate reductase in particular), under aerobic conditions was hypothesized to occur by one or a combination of the following four mechanisms:

- Oxygen interferes directly with the enzymes thereby preventing transfer of electrons from the reductases to the appropriate electron acceptors.
- Extracellular oxygen affects the intracellular redox potential of the organism, thereby affecting the flow of electrons between electron transport complexes.
- Oxygen prevents insertion of the subunits of nitrate reductase into the cytoplasmic membrane, thereby preventing transport of electrons from electron transferring complexes to nitrate reductase.
- Oxygen affects the permeability of the cytoplasmic membrane, thereby regulating the movement of nitrate to nitrate reductase, and preventing electron transport from nitrate reductase to nitrate.

Each of these proposed mechanisms is discussed in turn.

<u>Inactivation of nitrogen oxide reductases through direct interference by oxygen</u>

A mechanism by which oxygen could inactivate the nitrogen oxide reductases is through direct attachment to, or reaction with the reductases, in a manner which prevents the transfer of electrons to the nitrogen oxide electron acceptors. However, in research into this effect no such mechanism has been found; as proof that oxygen does not act in this manner, workers have considered it sufficient to demonstrate that oxygen inhibits the reduction of the nitrogen oxidases in a manner other than through direct interference with the reductases, such as by the mechanisms listed above and described below.

<u>Inhibition of electron flow to nitrogen oxide reductases due to intracellular redox changes</u>

Payne (1973) suggested that inactivation of nitrogen oxide reductase results from

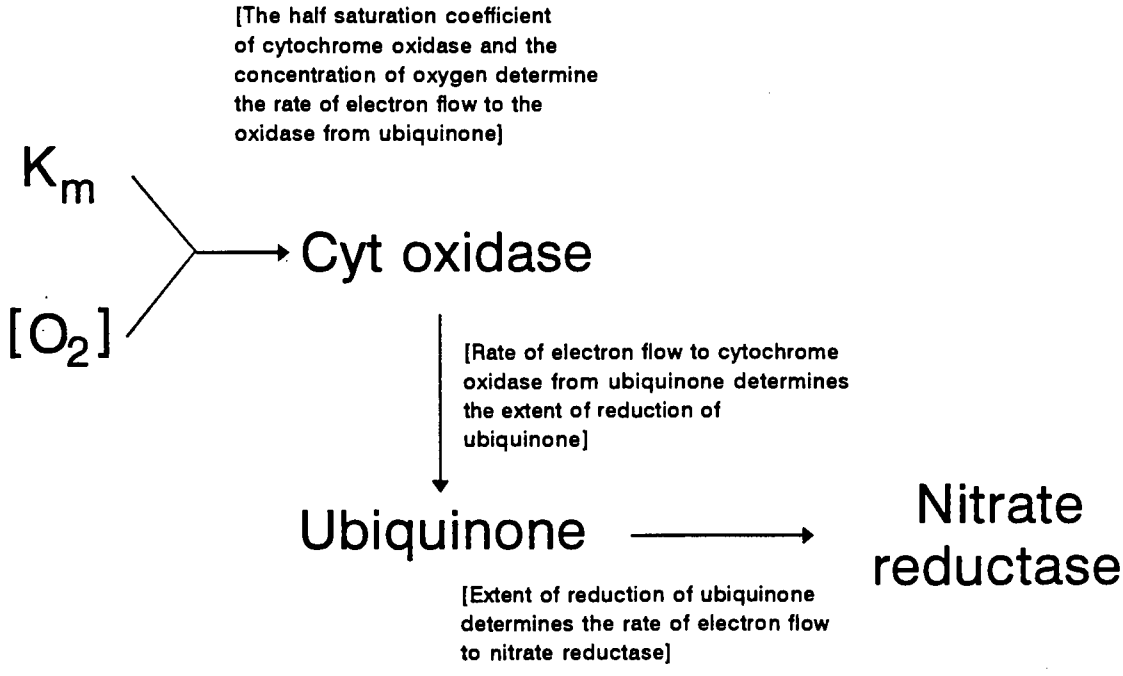

Fig 4.18: Proposed mechanism for the control of electron flow via the half saturation coefficient of the oxidase (k_m) and the redox state of ubiquinone (from Alefounder *et al.*, 1981).

- The addition of transmembrane nitrate carriers such as benzyl or heptyl viologen radicals caused nitrate reduction under aerobic conditions with *E. coli*. Without such carriers, nitrate reduction did not occur under aerobic conditions (Noji and Taniguchi, 1987).

- Nitrate reduction did not occur in intact cells of *Ps. aeruginosa* at low concentrations of oxygen, but in cell extracts in which the cytoplasmic membrane was destroyed, nitrate reduction was observed (Hernandez and Rowe, 1987, 1988).

These experiments provide strong evidence that under aerobic conditions the membrane becomes impermeable to nitrate (i.e. restricts the movement of nitrate across the membrane) thereby limiting nitrate reduction.

An additional finding in the third experiment was that when the membrane was made permeable with Triton X–100, nitrate was reduced to nitrite intracellularly and when a certain nitrite concentration was attained, electron flow to cytochrome oxidase was reduced and electron flow to the nitrogen oxide reductases increased. This finding has major implications with regard to aerobic respiration and is the subject of further scrutiny in Section 4.10 below which examines mechanisms of regulation of aerobic respiration.

An additional finding in the fifth experiment was that, in intact cells in which nitrate reduction was prevented at low concentrations of oxygen, nitrite reduction was not inhibited, and at higher concentrations of oxygen, nitrite reduction was only partially inhibited. This finding is due to differences in position between the active sites of nitrate and nitrite reductase; the active site of nitrate reductase is situated on the cytoplasmic side of the membrane and the active site of nitrite reductase on the periplasmic side. The impermeability of the membrane to nitrate caused by oxygen, does not apply to nitrite because it is not necessary for extracellular nitrite to cross the membrane to the active site of its reductase. If the only source of nitrite is intracellular (i.e., from cytoplasmically reduced nitrate) then regulation of nitrate movement across the membrane by oxygen would represent a primary mechanism by which the whole denitrification pathway could be controlled. However, if an extracellular source of nitrite is available (e.g. from oxidation of ammonium by *Nitrosomonas* in a mixed culture such as activated sludge), then control of membrane permeability by oxygen will not regulate the reduction of

in Chapter 5. Accordingly, in the section below, research into this aspect will be reviewed. Because the inhibition of oxidase activity was a consequence of an increase in nitrite concentration, the interaction of nitrite with cytochrome oxidase will be investigated as a first step.

Mechanisms of inhibition of aerobic respiration by nitrite

The inhibition of oxidase activity by nitrite has been investigated by a number of workers, using different techniques. Using *Pa. denitrificans*, Kučera and Dadak (1983) examined electron flow under aerobic conditions to cytochrome oxidase and nitrite reductase. An uncoupler was added to reduce the transmembrane potential of the cytoplasmic membrane, allowing access of nitrite to cytochrome oxidase. After addition of the uncoupler, electron flow to cytochrome oxidase decreased and electron flow to nitrite reductase increased, indicating that the mechanism of inhibition requires the access of nitrite to the cytoplasmically placed cytochrome oxidases.

However, without any changes to the membrane, Yang (1985) measured an inhibition of oxidase activity in *Ps. aeruginosa* following the addition of nitrite but no inhibition following the addition of nitrate. The result of non–inhibition with nitrate can be explained by the necessity for nitrate to cross the membrane but given that the inhibitory nitrite was not produced intracellularly but was added extracellularly, a means of traversing the membrane would also be required for nitrite to reach and inhibit cytochrome oxidase. Such a result would tend to indicate that oxidase inhibition is due to an effect of nitrite, but not due to nitrite *per se*. Further evidence for this view was provided in the following experiment.

Kučera *et al.* (1986b) investigated the effect of increasing electron flow to cytochrome oxidase and nitrate reductase in *Pa. denitrificans* through the addition of the artificial electron donor, ascorbate and a chemical TMPD (referred to as a mediator for its role in selectively increasing the flow of electrons to cytochrome oxidase and nitrite reductase to ensure the availability of electrons to nitrite reductase under aerobic conditions). In the absence of nitrite, oxidase activity increased with increased concentration of TMPD, but with nitrite present, oxidase activity initially increased with increased concentration of TMPD but decreased above a certain concentration of TMPD. The authors suggested that this decrease in oxidase activity was caused by the production of an inhibitory but unidentified intermediate resulting from the reduction of nitrite, possibly one of the

giving no indication of the inhibitory nature of nitric oxide (being bound to ferricytochrome *c* it was unable to interact with cytochrome oxidase), the experiment demonstrates that the product of the reduction of nitrite under aerobic conditions *is* nitric oxide.

From a hypothetical viewpoint, a concern with the proposal for nitric oxide as an inhibitory species was that its extremely reactive nature (with oxygen in particular) would not allow it to be present in sufficient quantity intracellularly to account for the degree of inhibition measured in laboratory studies (Parsonage *et al.*, 1985). In this regard Carr and Ferguson (1990b) demonstrated that under anoxic conditions the nitric oxide reductase of *Pa. denitrificans* maintained the steady–state concentration of dissolved nitric oxide sufficiently low that reaction with oxygen was insignificant, but sufficiently high that reaction with the active site of cytochrome oxidase would inhibit oxygen reduction.

Concerning the biochemical mechanism of inhibition of oxidase activity by nitric oxide; although the experimental work described above demonstrated that nitric oxide is the inhibitory species, the work did not describe the mechanism of inhibition. Four possible mechanisms are outlined and discussed below:

(1) Under aerobic conditions, electrons are directed away from cytochrome oxidase to the nitrogen oxide reductases by an intracellular redox effect due to the presence of nitric oxide. Kučera *et al.* (1983a) proposed that under anoxic conditions electron distribution between reductases is effected by redox control exerted by the concentration of the denitrification intermediates, and proposed that control of nitrate reduction by oxygen is effected by the same mechanism, thereby making it feasible that inhibition of oxidase activity by nitric oxide also occurs by the same mechanism.

(2) Nitric oxide accepts electrons directly from cytochrome oxidase, thereby reducing the transfer of electrons from cytochrome oxidase to oxygen; However, enzymes have a conformation specific to their oxidizing molecule (Robertis and Robertis, 1980) and it is unlikely that nitric oxide would have the correct conformation to effect electron transfer from cytochrome oxide.

(3) Nitric oxide reacts with dissolved intracellular molecular oxygen, thereby preventing oxygen attaining its site of reduction at cytochrome oxidase.

4.11 AEROBIC DENITRIFICATION

One of the principles arising from the work on inhibition of aerobic respiration is that whereas nitrate respiration cannot occur under aerobic conditions, nitrite reduction can. This is because nitrate reductase is situated on the cytoplasmic side of the membrane and oxygen makes the membrane impermeable to nitrate movement, whereas nitrite reductase is on the periplasmic side of the membrane and nitrite is not required to cross the membrane. Accordingly, nitrite can be reduced under aerobic conditions. Although aerobic denitrification has been an area of some debate the literature reports its occurrence among a great diversity of facultative organisms (Skerman *et al.*, 1951; Mechsner and Wuhrmann, 1963; Hernandez and Rowe, 1987; Robertson and Kuenen, 1983, 1984a,b, 1990). However the majority of literature is concerned with aerobic denitrification of nitrate, not nitrite, and it appears that denitrification of nitrate in those cases is attributable to organism tolerance of reduced oxygen levels, not redirection of electrons to nitrite reductase due to inhibition of cytochrome oxidase by nitric oxide. In contrast Krul (1976) in examining the effect of nitrate, nitrite, and nitric oxide on the oxygen uptake of an unidentified organism from activated sludge noted aerobic denitrification of nitrate and nitrite to nitric oxide but found the phenomenon difficult to explain. The aspect of aerobic denitrification forms an integral part of the biochemical model postulated in Chapter 5 for facultative organism respiration.

4.12 SUMMARY

In this Chapter a review of literature concerned with the respiration of facultative organisms was conducted in two parts: Part I reviewed the biochemical mechanisms involved in utilization of substrate for aerobic and anoxic respiratory processes, and the effect of the presence of oxygen, the absence of oxygen, and the presence of nitrogen oxides, on synthesis of the aerobic and anoxic electron transferring complexes of the ETP. Part II reviewed the mechanisms which initiate, regulate, and terminate aerobic and anoxic respiration.

To summarize briefly, the major conclusions of the review are:

From Part I;

- An electron transport pathway (ETP) can be described which can serve as a general pathway for facultative aerobic organisms, representing the major electron transferring complexes and sequences of these organisms.

CHAPTER 5

METABOLIC BEHAVIOUR OF FACULTATIVE AEROBIC ORGANISMS UNDER AERATED/UNAERATED CONDITIONS

ABSTRACT

A model is outlined for the major biochemical respiratory mechanisms of a conceptualized heterotrophic facultative organism (representing the heterotrophic facultative mass of activated sludge) subjected to four different sets of conditions; steady–state aerobic, steady–state anoxic, steady–state aerobic changed to anoxic, and steady–state anoxic/anaerobic changed to aerobic. Proposals and implications of the model regarding the effect of these conditions and changes between conditions on respiration under aerobic and anoxic conditions are tested experimentally and verified with special aerobic batch tests on sludges from IAND, 2RND, MUCT, continuous aerobic, and continuous anoxic systems fed municipal sewage.

PART I: A CONCEPTUAL BIOCHEMICAL MODEL

5.1 INTRODUCTION

In Chapter 4 a review was presented of the biochemical respiratory pathways and mechanisms operative in facultative heterotrophic organisms under a variety of environmental conditions. The review established that research has been conducted almost entirely with pure cultures of a number of organisms selected as representative of the main classes of heterotrophs, i.e. obligate aerobes, facultative aerobes which reduce nitrate, and facultative aerobes which denitrify. However, in nature, organisms are virtually never found in pure culture – they always exist in mixed cultures of unknown microbial constitution. An example of such a mixed culture is the microbial mass that develops in the activated sludge system. In the nutrient removal activated sludge sewage treatment process the microbial population is subjected to cyclic aerated and unaerated conditions. As a consequence of exposure to the sequences of aerated/unaerated conditions in these systems, it can be assumed that the microbial population will include a significant fraction of facultative heterotrophic organisms which have a capacity for substrate utilization under both aerated and unaerated conditions. With this assumption as a basis, and from the knowledge gained from the review of research on pure cultures, the overall objective of this investigation is to develop a microbiological/biochemical model that will allow the macroscopic biological responses observed in activated

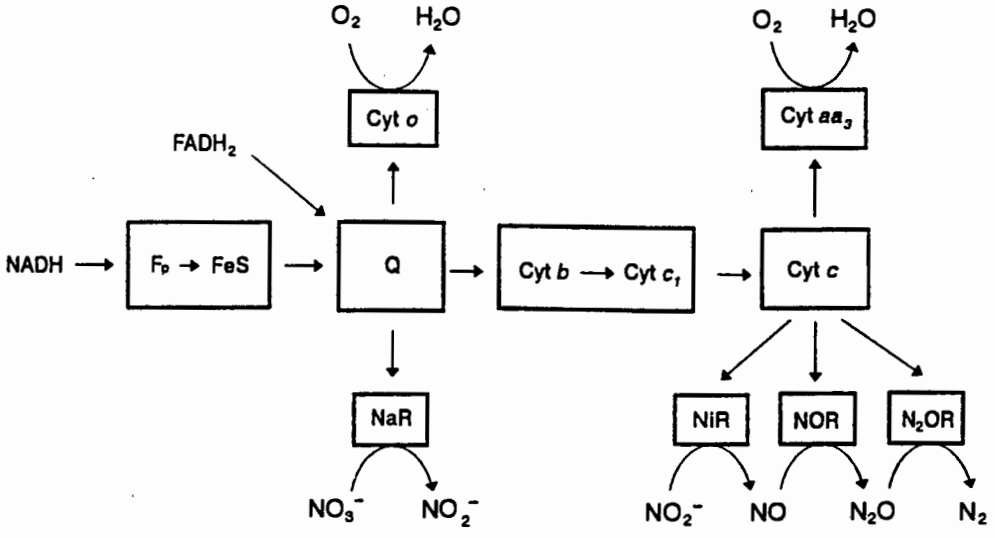

Fig 5.1: The electron transport pathway (ETP) for a conceptualized facultative heterotrophic organism as representative of the facultative heterotrophic organism mass in activated sludge [modification of pathway proposed by Ferguson (1982) for *Pa. denitrificans*].

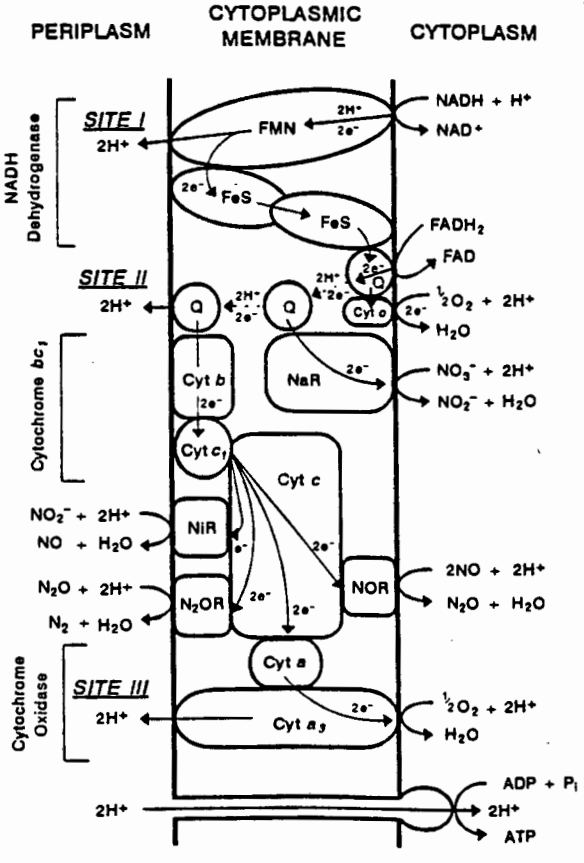

Fig 5.2: The electron transport pathway (ETP) for the conceptual facultative organism, in which all of the electron transferring complexes are present and illustrated with respect to the cytoplasmic membrane.

Electron and proton production and transport preceding the processes associated with the cytoplasmic membrane ETP

Each of the six parts of the model describe the transport of electrons, protons, and electron acceptors in processes associated with the ETP under aerobic or anoxic conditions. Other processes which precede these, such as the biochemical processes which result in the production of electrons and protons (captured by NADH) are common to both aerobic and anoxic respiration; these processes have been described fully in Part I of Chapter 4, and the descriptions are not repeated here. The role of NADH in the respiratory process is to serve as the link between electron/proton production and the ETP. NADH transports protons and electrons generated in the catabolic processes associated with the tricarboxylic acid (TCA) cycle to ETP complexes. These complexes extrude the protons to the periplasm, indirectly resulting in the production of energy, and transfer the electrons to ETP complexes, which in turn transfer them to one of the terminal electron acceptors, oxygen under aerobic conditions and nitrate or nitrite under anoxic conditions. The rate of electron transfer to the terminal electron acceptors is limited by the rate of production of NADH in the TCA cycle (Lehninger, 1975)[1].

Each of the six parts of the biochemical model is discussed in turn below.

Part 1 – Electron and proton transport from NADH to FMN, the FeS complexes and ubiquinone under both aerobic and anoxic conditions

The ETP complexes, NADH dehydrogenase, the FeS complexes, and ubiquinone are common to both aerobic and anoxic conditions. Under both aerobic and anoxic conditions, NADH transfers a pair of electrons and two protons (one originating from the cytoplasm) to the NADH dehydrogenase complex which comprises flavomononucleotide and FeS complexes. In this transfer two protons are removed from the cytoplasm and translocated to the periplasm at the first proton–pumping (energy conserving) site, Site I. Ubiquinone accepts a pair of electrons from the NADH dehydrogenase complex and two protons from the cytoplasm, mediates the transfer of electrons to the complexes involved in aerobic or anoxic respiration, and transports the protons to the periplasm at the second proton–pumping site, Site II. Thus, the first two sites (Sites I and II) are common to aerobic and anoxic respiration; a difference in energy production between aerobic and anoxic

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[1] As indicated in Chapter 4, electrons supplied to the ETP originate from both NADH and $FADH_2$. However, for simplification, where mention is made of the source of electrons, it is assumed to be NADH.

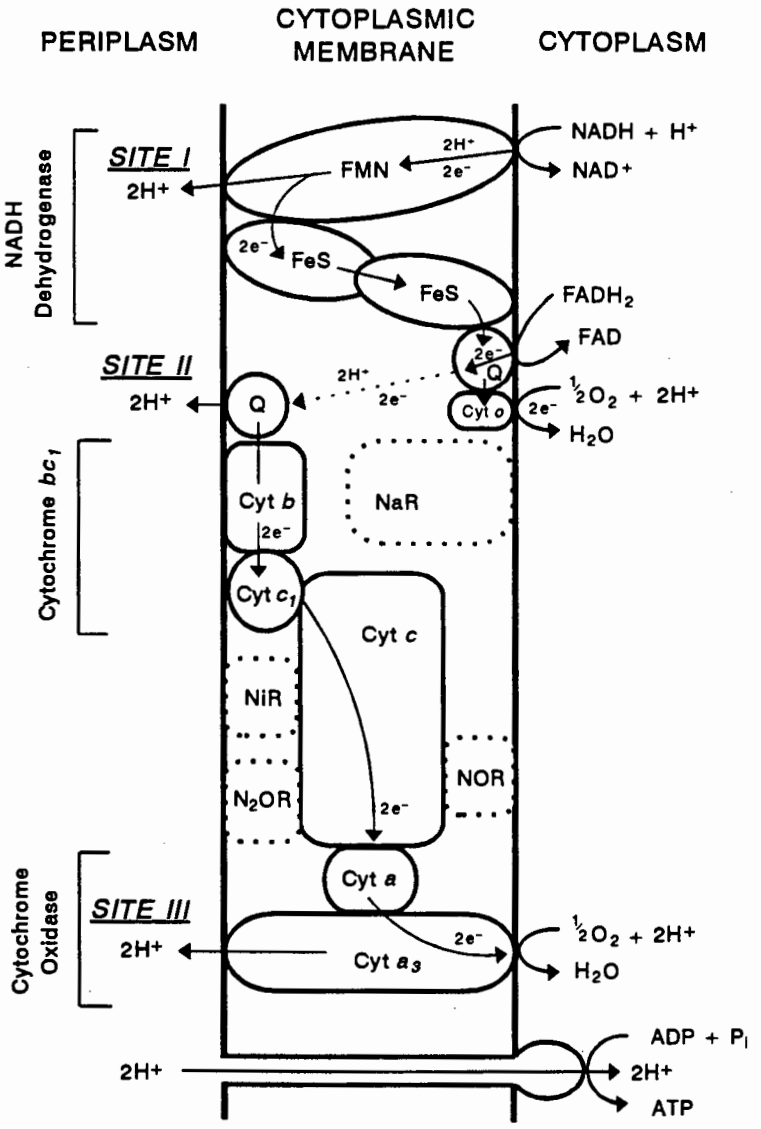

Fig 5.3: The ETP of the conceptualized facultative organism under aerobic conditions with dissolved oxygen (DO) $\geq$ 0,5 mgO/ℓ.

then to ubiquinone, but a smaller fraction of the electrons pass to the oxidases and a larger fraction pass to the reductases than at DO > 0,5 mgO/ℓ, and of the electrons which do pass to the oxidases, a greater proportion pass to cytochrome *o* than to cytochrome aa_3. The electrons which do not pass to the oxidases pass to the denitrifying enzymes, the nitrogen oxide reductases. Because of their low level of synthesis the reductases have only a very small electron transferring capacity. Within the range of low DO examined here, a priority in the order of reductase synthesis is established. At the higher concentrations of DO in the low DO range, only nitrate reductase is synthesized, but as the DO concentration decreases to 0,2 mgO/ℓ, each of the successive reductases of the denitrifying pathway is synthesized, i.e. nitrite–, nitric oxide–, and nitrous oxide reductase.

Part 3 – Anoxic conditions

The anoxic condition is defined as one in which oxygen is absent and nitrate or nitrite, or both, are present. Under anoxic conditions in the presence of the electron acceptors nitrate and nitrite, the status of the complexes of the ETP present in a facultative organism are shown in Fig 5.4. All of the nitrogen oxide reductases are present (Payne 1973), and also the oxidase cytochrome *o*, but the oxidase cytochrome aa_3 either is not present (Gray *et al.*, 1966; Lam and Nicholas, 1969c; Sapshead and Wimpenny, 1972), or is present at a low level (Alefounder *et al.*, 1981). The reduction of the nitrogen oxides can be best modelled by describing the sequential steps involved in the formation of one molecule of dinitrogen (N_2) from nitrate. Nitrate enters the organism through the cell wall and crosses the cytoplasmic membrane via one of two mechanisms; (i) with a proton (H^+), in a symport system which initiates nitrate reduction (Boogerd *et al.*, 1983a), or (ii) in exchange for an outgoing nitrite molecule in a nitrate/nitrite antiport system which functions once the process of nitrate reduction has been established by the first mechanism.

With nitrate present at nitrate reductase, electrons associated with NADH produced in the TCA cycle are passed via the NADH dehydrogenase complex to ubiquinone and then to nitrate reductase, where nitrate serves as electron acceptor and is reduced to nitrite on the cytoplasmic side of the membrane (John, 1977; Alefounder and Ferguson, 1980) at the catalytic site of nitrate reductase (the α subunit) (Chaudhry and MacGregor, 1983b). Reduction of nitrate (NO_3^-) to nitrite (NO_2^-) is as follows:

$$2H^+ + 2e^- + NO_3^- \rightarrow NO_2^- + H_2O \qquad (5.2)$$

From the cytoplasmic side, the nitrite molecule crosses the membrane via the NO_3^-/NO_2^- antiport system to the nitrite reductase complex at the periplasm (Wood, 1978; Alefounder and Ferguson, 1980). With nitrite present at nitrite reductase, electrons associated with NADH produced in the TCA cycle are passed via the NADH dehydrogenase complex to ubiquinone, to the cytochrome bc_1 complex, to cytochrome *c* and then to nitrite reductase, where nitrite produced from the reduction of nitrate and any other extracellularly produced nitrite are reduced to nitric oxide (NO) at the nitrite reductase complex as follows:

$$2H^+ + e^- + NO_2^- \rightarrow NO + H_2O \qquad (5.3)$$

From the periplasmic side of the membrane, the product nitric oxide, passes back to the cytoplasmic side of the membrane to the nitric oxide reductase complex (Heiss *et al.*, 1989; Carr *et al.*, 1989). With nitric oxide present at the active site of nitric oxide reductase, electrons associated with NADH produced in the TCA cycle are passed through the same complexes as for nitrite reduction to cytochrome *c* and then to nitric oxide reductase where nitric oxide produced from the reduction of nitrite is reduced to nitrous oxide (N_2O) as follows:

$$2H^+ + 2e^- + 2NO \rightarrow N_2O + H_2O \qquad (5.4)$$

From the cytoplasmic side, the nitrous oxide molecule then passes across the membrane to the nitrous oxide reductase complex on the periplasmic side of the membrane (Boogerd *et al.*, 1981). With nitrous oxide present at nitrous oxide reductase, electrons associated with NADH produced in the TCA cycle are passed through the same complexes as for nitrite reduction and nitric oxide reduction, and then to nitrous oxide reductase where nitrous oxide is reduced to dinitrogen (N_2) as follows:

$$2H^+ + 2e^- + N_2O \rightarrow N_2 + H_2O \qquad (5.5)$$

The endproduct of the reaction, dinitrogen, is released through the cell wall to the external medium.

The denitrification processes as described above are somewhat simplistic in that the

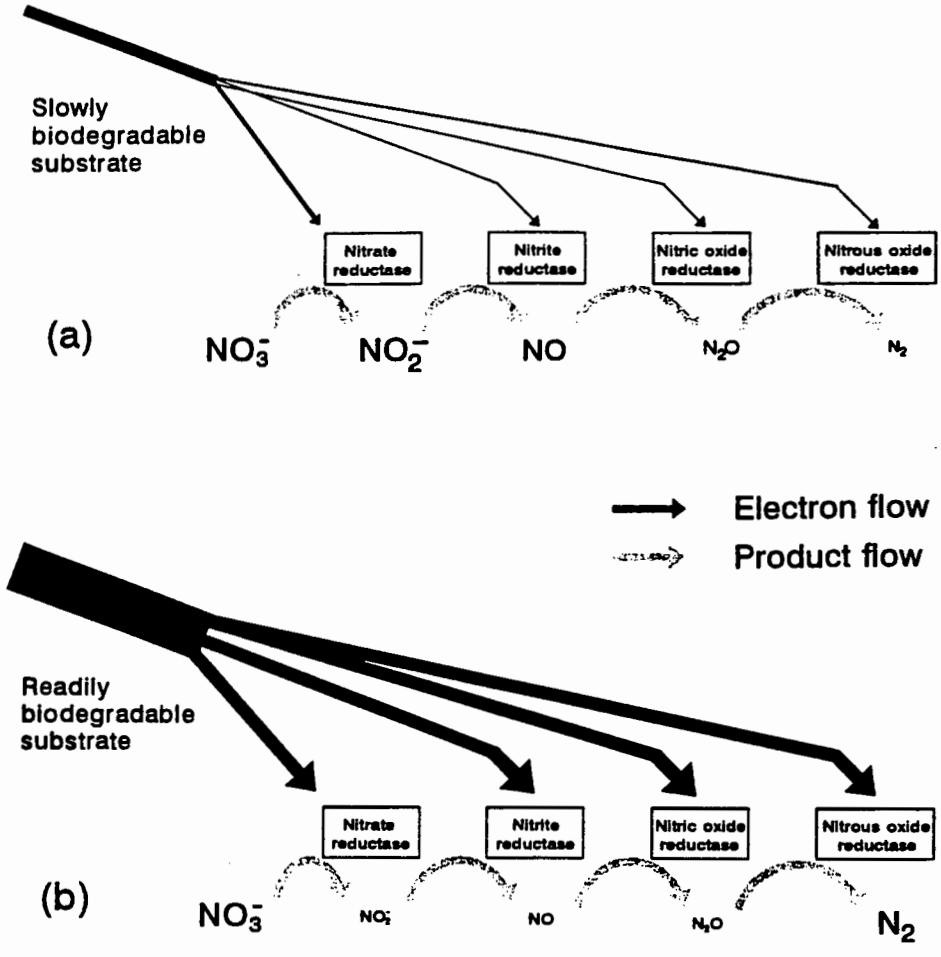

Fig 5.5: Diagrammatic representation of the kinetics of the denitrification pathway indicating rates of electron flow and steady–state intracellular levels of the nitrogen oxides under anoxic conditions with adequate nitrate present and the ETP supplied with; (a) a low rate of electron supply (from slowly biodegradable substrate), and (b) a high rate of electron supply (from readily biodegradable substrate).

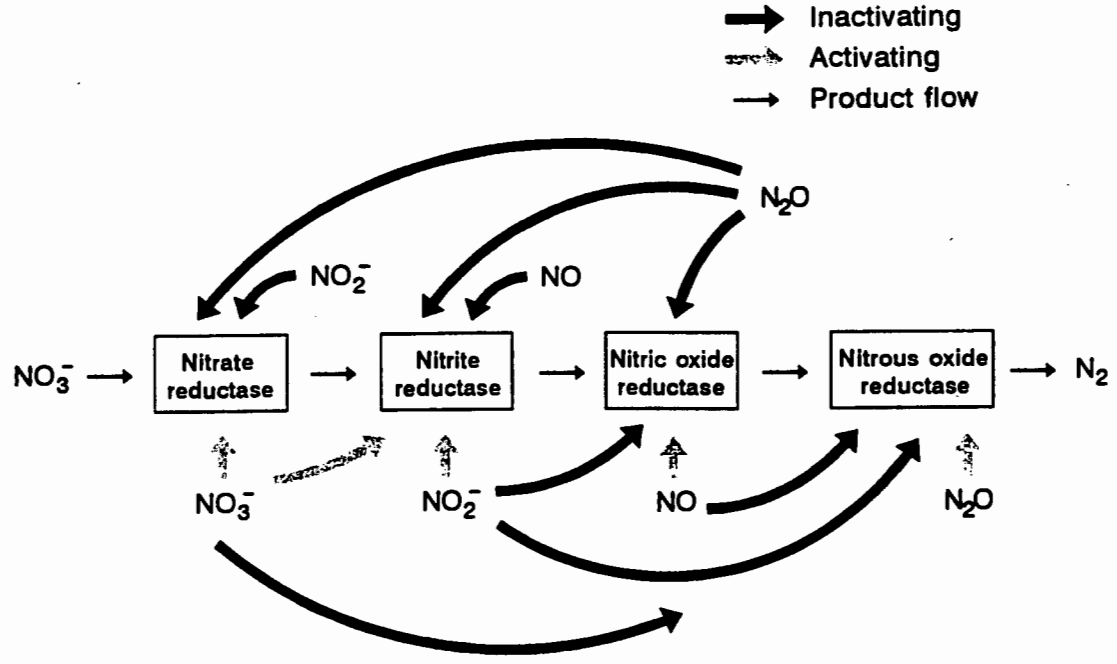

Fig 5.6: Summary of the effect of the nitrogen oxides on the activities of the nitrogen oxide reductases of the denitrifying electron transport pathway (ETP) and the direction of product (nitrogen oxide) flow.

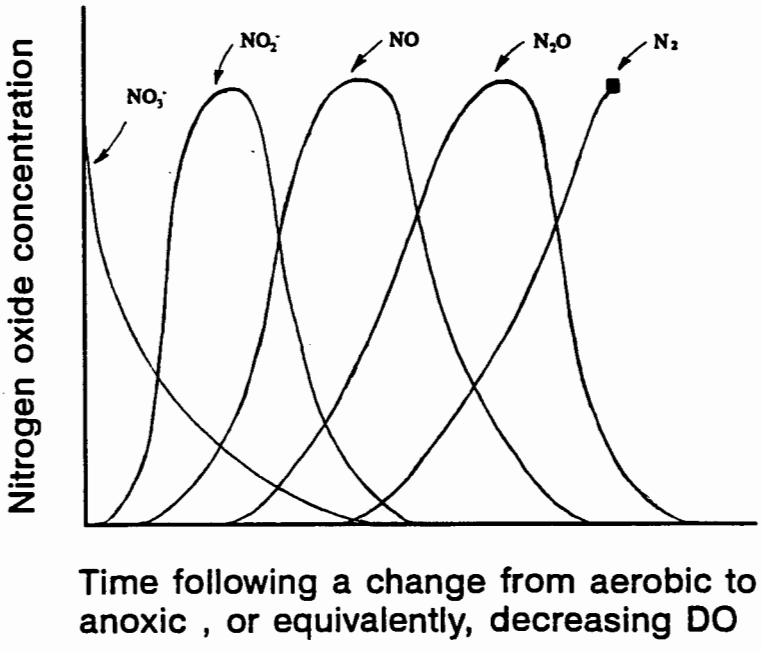

Fig 5.7: Sequential formation, with time (or equivalently dissolved oxygen concentration) of the nitrogen oxides, nitrate, nitrite, nitric oxide, and nitrous oxide for a facultative organism under steady-state aerobic conditions transferred to anoxic conditions with nitrate present (redrawn from Tiedje, 1985).

Part 6 – Anoxic conditions changed to aerobic conditions

In Parts 2 and 3 it was ascertained that different complexes are synthesized under steady–state anoxic conditions (Fig 5.4) and steady–state aerobic conditions (Fig 5.3), and that the reactions mediated by these complexes are also different. This implies that in undergoing a change from steady–state anoxic with one or both of the ionic nitrogen oxides nitrate or nitrite present, to aerobic with oxygen present, the status of the ETP changes with time from that described by Fig 5.4 to that described by Fig 5.3. At the onset of aerobic conditions four major changes occur in the complexes and transport processes associated with the ETP. Briefly, these are:

- reduction in nitrogen oxide reductase activities,
- reduction in permeability of the cytoplasmic membrane to nitrate,
- cessation of synthesis of nitrogen oxide reductases, reduction in the synthesis of cytochrome oxidase *o*, and initiation of synthesis of cytochrome aa_3,
- interaction of nitric oxide with the cytochrome oxidases.

In combination, the four changes contribute to or are implicated in a mechanism which results in the inhibition of aerobic respiration following a transition from anoxic to aerobic conditions, and the four changes are dealt with in the order in which they affect aerobic respiration after the transition. Each change is dealt with in turn.

Changes in nitrogen oxide reductase activities

Oxygen inhibits the activities of the nitrogen oxide reductases in the order corresponding to the reductases for the increasingly less reduced nitrogen oxides, i.e. the nitrous oxide –, nitric oxide –, nitrite –, and nitrate reductases (Hochstein *et al.*, 1984), either with time (e.g. nitrous oxide reductase activity is inhibited prior to nitric oxide reductase at any constant oxygen concentration) or with increase in oxygen concentration (e.g. nitrous oxide reductase activity is inhibited at a lower oxygen concentration than is nitric oxide reductase activity). This is illustrated in Fig 5.8 which describes the effect of oxygen on the activities (and synthesis) of the reductases. The result of exposure to aerobic conditions is the accumulation of the nitrogen oxides in the order, nitrous oxide, nitric oxide, nitrite, and nitrate. The mechanism by which oxygen affects nitrogen oxide reductase activity is through redirection of electrons, from the reductases to the oxidases as a consequence of the greater redox potential for electron flow created by the presence of oxygen at the oxidases than by the presence of the nitrogen oxides at the reductases (Alefounder

et al., 1981).

Change in the permeability of the cytoplasmic membrane

Oxygen induces a change in the permeability of the cytoplasmic membrane such that the movement of nitrate to its reductase is prevented (John, 1977; Alefounder and Ferguson, 1980; Kučera *et al.*, 1983b; Noji and Taniguchi, 1987). This effect is apparently specific to the movement of nitrate. Assuming that under steady-state anoxic conditions nitrate movement across the membrane occurs via an NO_3^-/NO_2^- antiport mechanism, it is assumed that impermeability of the membrane to nitrate movement refers to the effect of oxygen in preventing operation of the antiport mechanism.

Change in the synthesis of the anoxic and aerobic enzyme complexes

Oxygen prevents the synthesis of the nitrogen oxide reductases but induces the synthesis of the principal oxidase cytochrome aa_3. The synthesis of the alternative oxidase cytochrome *o* continues under aerobic conditions (Lam and Nicholas, 1967; Newton, 1967; Lam and Nicholas, 1969c) but is synthesized at only 30% of the level that it is synthesized under anoxic conditions. It is considered that synthesis of cytochrome aa_3 occurs at a rate commensurate with the rate of degradation of cytochrome *o*,[2] such that the nett level of available cytochrome oxidase (cytochrome *o* + cytochrome aa_3) is at the least, *maintained* under aerobic conditions. Under aerobic conditions, electron flow to the oxidases is utilized in two processes; (i) for the reduction of oxygen and, (ii) for the synthesis of cytochrome aa_3. It is unclear from the literature as to the period of time required for the synthesis of cytochrome aa_3 to steady-state level, but from experimental work conducted in this investigation, it would appear that given a high rate of supply of electrons, a period of 6–8 hours is necessary and given a low rate of supply of electrons, a number of days may be required. The mechanism by which oxygen represses synthesis of nitrate reductase is through oxygen binding to a protein (which regulates nitrate reductase synthesis) causing a conformational change, enabling the protein to bind to a segment of DNA specific to synthesis of nitrate reductase, thereby preventing its synthesis (Stouthamer 1988).

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[2] Modelling the heterotrophic facultative sludge mass as a surrogate heterotrophic facultative organism necessitates the degradation with time of cytochrome *o*. In individual organisms in the sludge mass, it is likely that the level of cytochrome *o* under anoxic conditions does not degrade, but that "new" organisms have a significantly lower level of cytochrome *o*, yielding a lower nett level for the sludge mass with time, modelled by a decreasing level of cytochrome *o* in the surrogate organism.

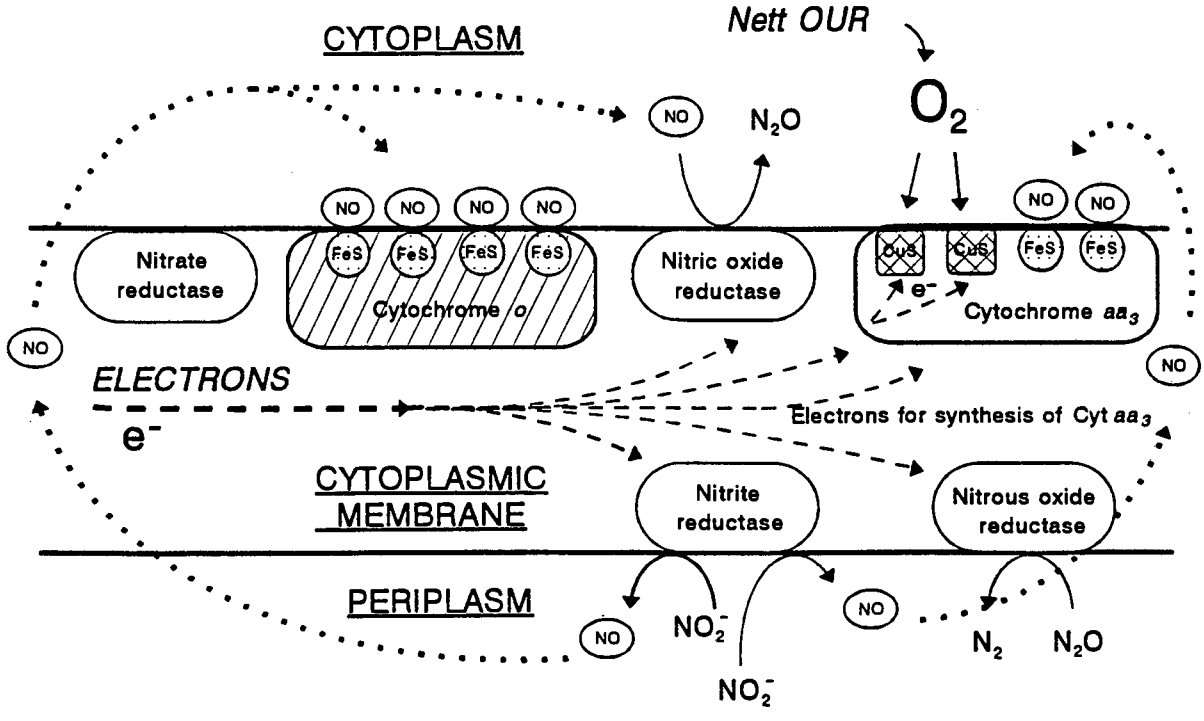

Fig 5.9: Interactions about the cytoplasmic membrane between nitric oxide and cytrochrome oxidase under aerobic conditions following anoxic conditions with nitrite present, indicating the mechanisms resulting in inhibition of aerobic respiration.

(i) *Readily biodegradable substrate absent and nitrite present under sequential anoxic and aerobic conditions:*

Figures 5.10a to 5.10c illustrate the interactions involving electrons, nitrite reductase, the oxidases (cytochrome *o* and cytochrome aa_3), nitric oxide and oxygen, which occur about the cytoplasmic membrane with time with a slowly biodegradable substrate as electron source and with nitrite *present* at the start of aerobic conditions following steady-state anoxic conditions with readily biodegradable substrate absent and nitrite present.

Under the preceding anoxic conditions in which slowly biodegradable substrate supplies electrons to reduce nitrite, nitric oxide accumulates (Fig 5.10a). As shown in Fig 5.5, the kinetics of the denitrification pathway indicate that with slowly biodegradable substrate the rate of formation of nitric oxide is greater than its rate of reduction and as a result it accumulates. Under the subsequent aerobic conditions with slowly biodegradable substrate and nitrite present, nitric oxide interacts with the FeS centres of the cytochrome *o* and cytochrome aa_3 complexes, preventing the transfer of electrons to oxygen. Electrons are then directed to the electron-transferring site at which the next highest redox potential is established - nitrite reductase. (Since the cytoplasmic membrane is impermeable to nitrate movement, nitrate cannot pass to nitrate reductase and only a low redox potential is established at that reductase). At nitrite reductase, nitrite is reduced to nitric oxide, which passes across the membrane and interacts with the FeS centres of any uninhibited cytochrome *o*, and any newly synthesized cytochrome aa_3 (Fig 5.10b). Thus, under conditions in which cytochrome oxidase is inhibited, nitrite is reduced to nitric oxide under aerobic conditions (i.e. aerobic denitrification). With extended time under aerobic conditions, inhibition is relieved through two processes: (1) new cytochrome aa_3 is synthesized (the rate of synthesis is low due to a low availability of electrons from slowly biodegradable substrate), and since nitric oxide does not interact with the CuS centres of the cytochrome aa_3 complex, these centres transfer electrons to the terminal electron acceptor oxygen, at a higher rate than the inhibited cytochrome *o*, (2) the nitrogen oxide reductases are degraded (see footnote 2), and less nitric oxide is produced. These changes are illustrated in Fig 5.10c, the consequence of which is an increased rate of aerobic respiration. *Rapid* relief of inhibition, through rapid synthesis of cytochrome aa_3 requires a high rate of electron supply, which can be

achieved only with readily biodegradable substrate.

(ii) *Readily biodegradable substrate and nitrite absent under sequential anoxic (anaerobic) and aerobic conditions:*

Figures 5.11a and 5.11b illustrate the interactions involving nitrite reductase, the oxidases (cytochrome *o* and cytochrome aa_3), nitric oxide, and oxygen which occur about the cytoplasmic membrane under aerobic conditions with time, with slowly biodegradable substrate as electron source, following anoxic/anaerobic conditions with slowly biodegradable substrate present and nitrite absent.

Under the preceding anoxic/anaerobic conditions, denitrification of nitrite is essentially complete and only a residual amount of nitric oxide remains (Fig 5.11a). Under the subsequent aerobic conditions the proportion of FeS centres which have not formed iron–nitric oxide complexes is high and aerobic respiration under such conditions is essentially uninhibited (Fig 5.11b). Extended aerobic conditions do not cause a change to the rate of aerobic respiration. Inhibition can be induced in such organisms through the imposition of a high external concentration of nitrite which leads to a high intracellular level of nitrite on the periplasmic side of the membrane. Since the active site of nitrite reductase is situated on the periplasmic side of the membrane a high redox potential is established at nitrite reductase such that electrons are attracted to the reductase and nitrite is reduced to nitric oxide. Nitric oxide then interacts with the FeS complexes of the oxidases and aerobic respiration is inhibited. Inhibition does not continue indefinitely with time; degradation of the nitrogen oxide reductases under aerobic conditions results in reduced levels and eventually the absence of nitric oxide, irrespective of the concentration of nitrite.

Inhibition *cannot* be induced through the imposition of a high external concentration of *nitrate*, since the active site of nitrate reductase is situated on the cytoplasmic side of the cytoplasmic membrane, and oxygen changes the permeability of the membrane, preventing access of nitrate to its reductase.

(iii) *Readily biodegradable substrate absent and nitrite present under anoxic conditions; readily biodegradable substrate and nitrite present under subsequent aerobic conditions:*

It was noted in (i) above that inhibition can be relieved by the further

synthesis of cytochrome aa_3, the CuS centres of which do not interact with nitric oxide. A high rate of synthesis requires a higher rate of supply of electrons than can be achieved with slowly biodegradable substrate. Figures 5.12a to 5.12d describe the interactions involving nitrite reductase, the oxidases (cytochrome *o* and cytochrome aa_3), nitric oxide, and oxygen, which occur about the cytoplasmic membrane under aerobic conditions with electrons supplied by readily biodegradable substrate.

Under the preceding anoxic conditions with readily biodegradable substrate absent and nitrate and nitrite present, nitric oxide accumulates to a high level as illustrated in Fig 5.12a (see also Fig 5.5). Under subsequent aerobic conditions with both readily biodegradable substrate and nitrite present, cytochrome oxidase is initially inhibited by the nitric oxide and electrons are redirected to nitrite reductase where nitrite is reduced to nitric oxide (Fig 5.12b). The high rate of electron supply from readily biodegradable substrate serves three purposes, (a) to supply electrons for synthesis of cytochrome aa_3, (b) to supply electrons for transfer to oxygen, and (b) to supply electrons for reduction of nitric oxide and other nitrogen oxides resulting in an increasing rate of aerobic respiration (Fig 5.12c). With time under aerobic conditions, the high rate rate of electron supply allows a high rate of cytochrome aa_3 synthesis, promoting an increased flow of electrons to that oxidase, the nitrogen oxide reductases are degraded, less nitric oxide is produced and inhibition is relieved (Fig 5.12d).

The nett result of these mechanisms is that inhibition induced by a high concentration of nitrite and slowly biodegradable substrate under anoxic conditions can be relieved under subsequent aerobic conditions by an adequate supply of electrons from readily biodegradable substrate.

(iv) *Readily biodegradable substrate and nitrite present under sequential anoxic and aerobic conditions:*

Sections (i) to (iii) above have dealt exclusively with the differences between respiration with readily and slowly biodegradable substrate under *aerobic conditions* following anoxic conditions with nitrite present or absent. The mechanisms associated with the use of readily biodegradable substrate under anoxic conditions have not been examined. Figures 5.13a and 5.13b describe the interactions involving electrons, nitrite reductase and nitrogen oxides

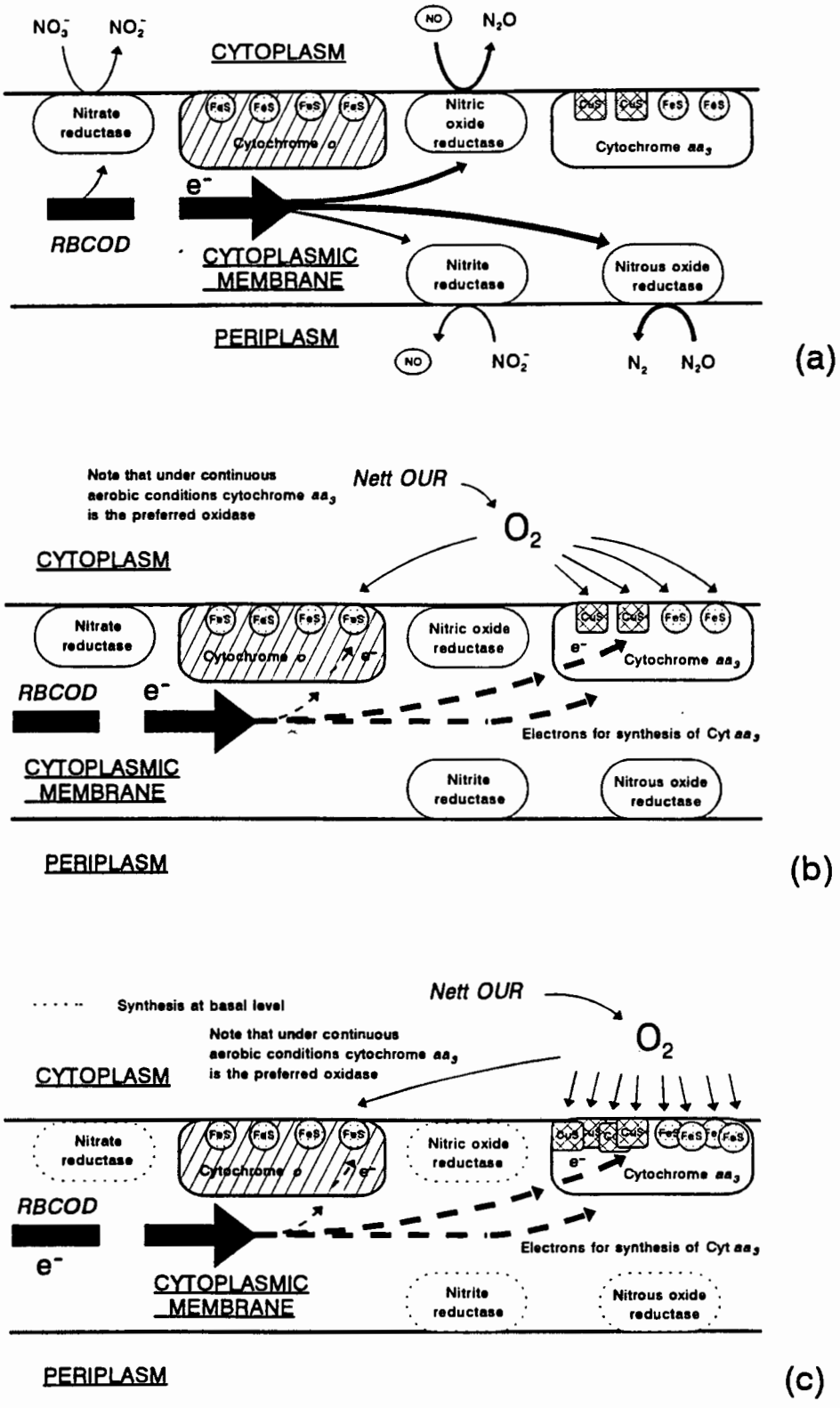

Fig 5.13: Interactions about the cytoplasmic membrane under (a) anoxic conditions followed by (b,c) aerobic conditions with nitrite present and readily biodegradable substrate (RBCOD) as electron source under both sets of conditions.

5.4 PROPOSALS AND IMPLICATIONS OF THE BIOCHEMICAL MODEL

(1) Effect of nitrite concentration under anoxic conditions on induction of inhibition of respiration under subsequent aerobic conditions

- During denitrification of nitrite under anoxic conditions (i.e. $NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$) with slowly biodegradable substrate, nitric oxide accumulates which inhibits utilization of oxygen under subsequent aerobic conditions.

- With nitrite absent under anoxic/anaerobic conditions, no accumulation of nitric oxide occurs and aerobic respiration will not be inhibited under subsequent aerobic conditions.

- An increase in the concentration of nitrite being denitrified with slowly biodegradable substrate under anoxic conditions gives rise to an increased oxygen utilization inhibition under subsequent aerobic conditions.

(2) Effect of nitrite and nitrate under aerobic conditions on induction of inhibition of aerobic respiration

- With nitrite added or present under aerobic conditions with only slowly biodegradable substrate present, inhibition of respiration will be induced.

- The extent of inhibition of aerobic respiration, induced by denitrification of nitrite with slowly biodegradable COD, will be proportional to the length of time nitrite is present due to low rates of electron flow to nitrite reductase.

- With nitrate added or present under aerobic conditions with only slowly biodegradable substrate present, inhibition of respiration will not be induced due to impermeability of the membrane to nitrate movement.

(3) Effect of substrate biodegradability under aerobic conditions on induction and relief of inhibition of aerobic respiration

- With only slowly biodegradable substrate present under aerobic conditions, nitrite added or present is denitrified and inhibition of respiration will be induced.

- Readily biodegradable substrate under aerobic conditions relieves inhibition of aerobic respiration induced by denitrification of nitrite with slowly

PART II: EXPERIMENTAL TESTING OF THE CONCEPTUAL BIOCHEMICAL MODEL

5.5 INTRODUCTION

In Part I a conceptual model was formulated for the biochemical mechanisms of aerobic–facultative heterotrophic organisms operative under various environmental conditions. The model was formulated from an amalgamation of (i) a literature review which highlighted the significant biochemical mechanisms of facultative aerobic organisms, especially with regard to interaction between the aerobic and anoxic respiration pathways of those organisms, (ii) hypotheses regarding the kinetics of denitrification under aerobic and anoxic conditions with readily biodegradable substrate present and absent, and (iii) results from experiments conducted with defined artificial substrate and municipal sewage.

A difficulty in testing the model is in the examination of organism behaviour on a level as fundamental as biochemical pathways. This problem was exacerbated by the limited facilities available in a wastewater treatment laboratory designed to examine extracellular macroscopic microbiological phenomena such as nitrification, aerobic substrate utilization rates, denitrification rates, phosphorus removal, and biomass production. Using such facilities, changes in intracellular biochemical processes such as enzyme synthesis and activity can be measured only indirectly, and as such, the experimental programme which follows can be considered as an attempt to accumulate an overwhelming body of circumstantial evidence for or against the hypothesized biochemical model. Experimental work to test the model was oriented at examining the proposals and implications, listed above.

It is important to note that just as the implications listed under categories (1), (2), and (3) are for organisms subjected to frequent exposure to anoxic–aerobic alternating conditions (such that both aerobic and anoxic respiratory enzymes are synthesized), so the experimental work to test the implications was conducted on sludge subjected to frequent anoxic–aerobic alternating conditions, i.e. a 2RND (MLE) system (System 9) with 3:1 a–recycle and 1:1 s–recycle, unless otherwise stated. For the implications listed under category (4), experimental work to test these was conducted on sludge subjected to steady–state aerobic and anoxic periods.

5.6 INHIBITION OF AEROBIC RESPIRATION IN ACTIVATED SLUDGE

A large number of experiments with aerobic batch tests fed municipal sewage were

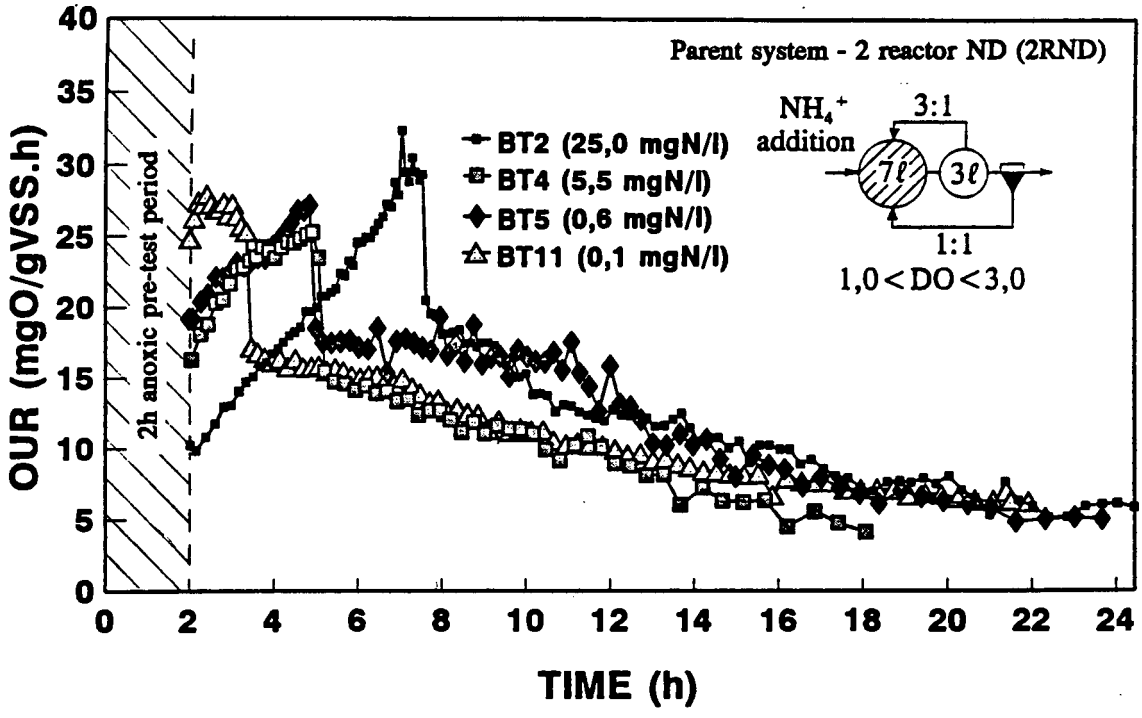

Fig 5.14: Oxygen utilization rate (OUR in mgO/gVSS.h) with time (h) for Batch Tests 2, 4, 5 and 11 with different concentrations of nitrite present at the onset of aerobic conditions.

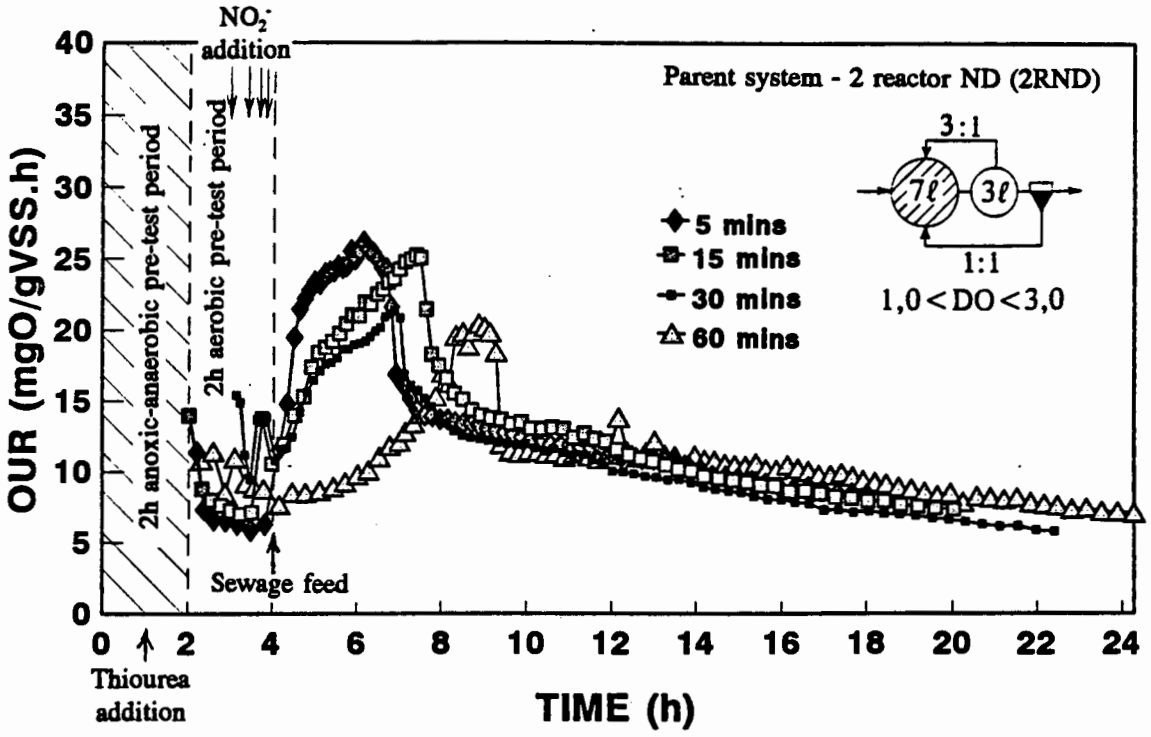

Fig 5.15: Oxygen utilization rate (OUR in mgO/gVSS.h) with time (h) for Batch Tests 13, 14, 15 and 16 with nitrite addition under aerobic conditions at different time periods before the addition of substrate.

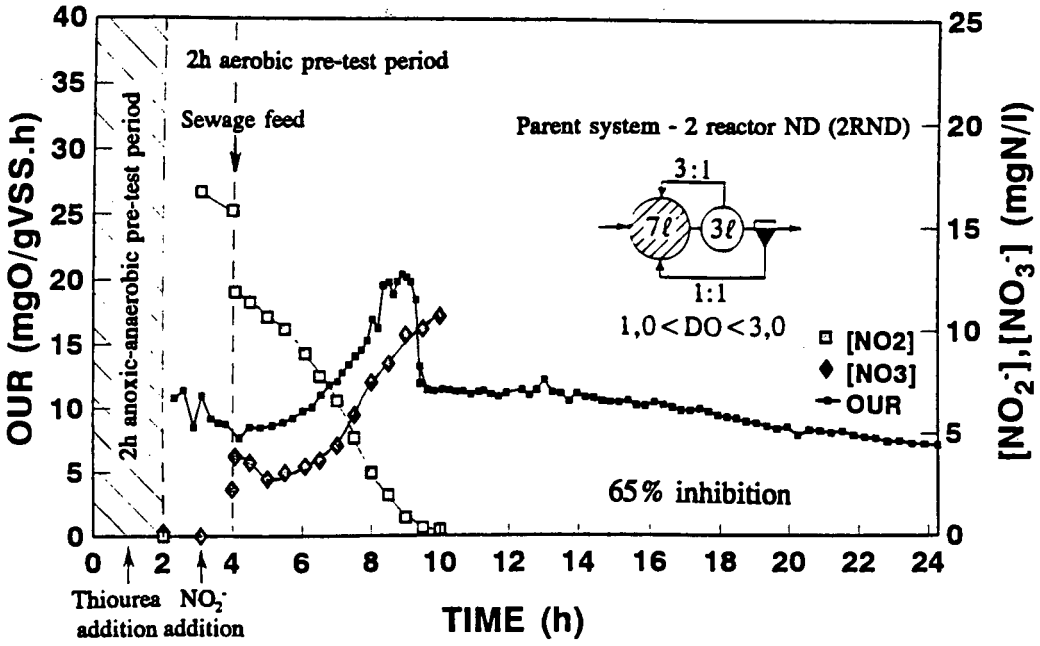

Fig 5.16: Oxygen utilization rate (OUR in mgO/gVSS.h) with time (h) for Batch Test 13 with nitrite added under aerobic conditions in the absence of readily biodegradable substrate.

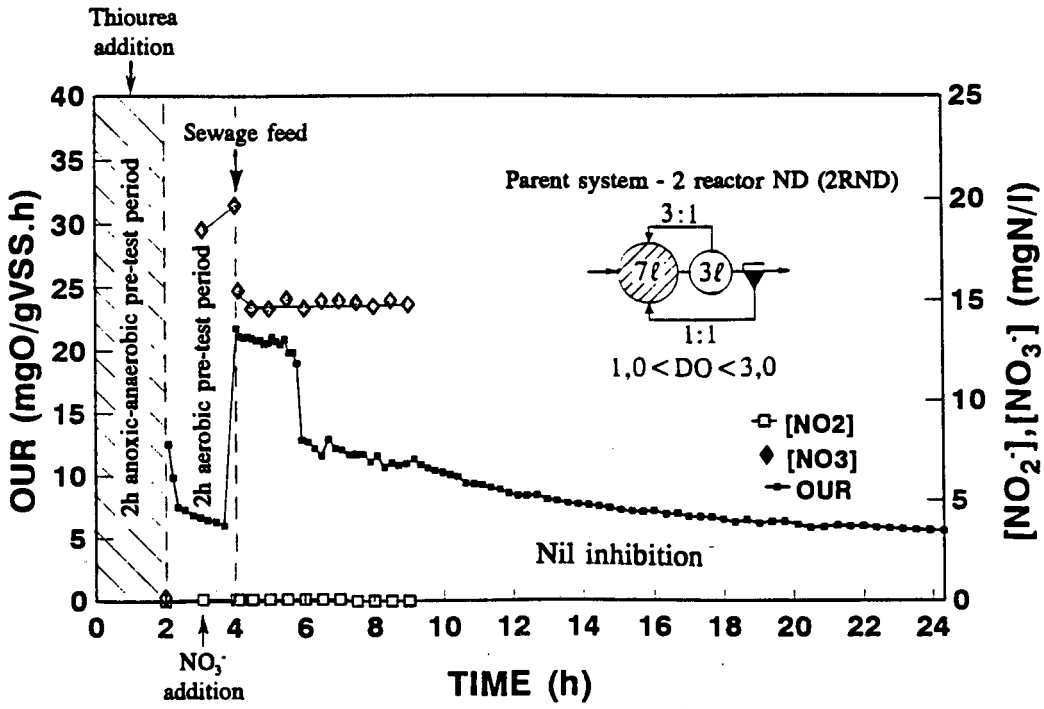

Fig 5.17: Oxygen utilization rate (OUR in mgO/gVSS.h) with time (h) for Batch Test 12 with nitrate added under aerobic conditions in the absence of readily biodegradable substrate demonstrating no induction of inhibition.

- ***With readily biodegradable substrate present under aerobic conditions, nitrite added will not be denitrified and inhibition will not be induced***

The biochemical model proposes that after a change from anoxic to aerobic conditions, when NADH supplies electrons in excess to the ETP, the intracellular concentration of nitric oxide is maintained low enough by nitric oxide reductase to prevent inhibition of aerobic respiration. When the supply of electrons from NADH is restricted, the intracellular concentration of nitric oxide increases because electrons flow preferentially to nitrite reductase to produce nitric oxide, than to nitric oxide reductase to reduce it; the increased nitric oxide concentration inhibits aerobic respiration.

From the foregoing batch tests it is apparent that addition of RBCOD relieves the inhibition effect, reflected in a steadily increasing OUR following the addition of substrate.

To test this aspect more directly, three batch tests were conducted; Batch Test 17 in which nitrite was added in the presence of readily biodegradable substrate and inhibition was not induced (Fig 5.18), Batch Test 1 in which no nitrite was added to the batch test and inhibition was not induced (Fig 5.19), and Batch Test 13 in which nitrite was added in the absence of readily biodegradable substrate and inhibition was induced, as described previously (Fig 5.16) and shown again to facilitate comparison of the tests. In Batch Test 17, nitrite was added about an hour after the addition of substrate and the OUR increased due to nitrification of nitrite. An alternative conclusion is that inhibition was induced, but that the decrease in OUR due to inhibition was offset by a larger increase in OUR due to nitrification. However, this would appear not to be the case, given that for BT13 (Fig 5.16) the addition of nitrite in the absence of readily biodegradable substrate had an oxygen requirement (for nitrification) of 3,0 mgO/gVSS.h, a requirement similar to that for the addition of nitrite in the presence of readily biodegradable substrate in BT17 (3,6 mgO/gVSS.h). These results would indicate that the increase in OUR following the addition of nitrite was due to nitrification only, not the nett result of inhibition and nitrification.

- ***Inhibition effect relieved on a sludge with readily biodegradable substrate will be re-induced in the absence of readily biodegradable substrate***

From the model, in the presence of an adequate supply of electrons, inhibition is relieved, and in the absence of an adequate supply of electrons, inhibition is induced

in the presence of nitrite. In the foregoing batch tests in which inhibition is relieved with readily biodegradable substrate, it is unclear as to whether relief is permanent or temporary. To examine this aspect, Batch Tests 7 and 8 were conducted, in which substrate was added to sludge in which nitrite and nitrate had been removed, as illustrated in Fig 5.20. The OUR profile indicates that the RBCOD was completely utilized after 2 hours and that the sludge was inhibition-free. About 20 hours after substrate addition, nitrite was added at a concentration of 6,0 $mgNO_2^--N/\ell$ and 5 minutes later substrate added. The presence of nitrite, slowly biodegradable substrate, but not readily biodegradable substrate in the 5 minute period prior to substrate addition induced inhibition, which took $4^1/_2$ hours to overcome. From the results of the experiments, it can be concluded that neither induction nor relief of inhibition is permanent, but is a function of the presence or absence of readily biodegradable substrate and nitrite. The results of the experiments are in agreement with the proposals of the model; relief and absence of inhibition occurs in the presence of an adequate supply of electrons (readily biodegradable substrate) and inhibition occurs or is induced in the presence of an inadequate supply of electrons (slowly biodegradable substrate) with nitrite present, irrespective of whether the sludge has previously been relieved of or was free of inhibition.

(4) <u>Inhibition of respiration is a general mechanism applicable to all sludges subjected to alternating anoxic-aerobic conditions irrespective of system configuration</u>

The inhibition tests described above were all conducted on sludges developed in a 2RND post denitrification (MLE) configuration with a 3:1 a-recycle and a 1:1 s-recycle. To ensure that inhibition of respiration is not specific to MLE configurations, sludges from a multi-reactor MUCT configuration and an intermittently aerated nitrification-denitrification (IAND) system were each subjected to the same conditions which induced inhibition in a sludge from an MLE system.

For the MUCT system, Batch Tests 22 and 24, illustrated in Figs 5.21 and 5.22 respectively, indicate that similar results are measured for inhibition of aerobic respiration for sludge from MUCT systems as for 2RND systems.

For the IAND system, Fig 5.23 describes the results of Batch Tests 20 and 21 in which nitrite was present and absent respectively during a 2 hour anoxic period prior to aeration and addition of substrate. The tests indicate that similar results

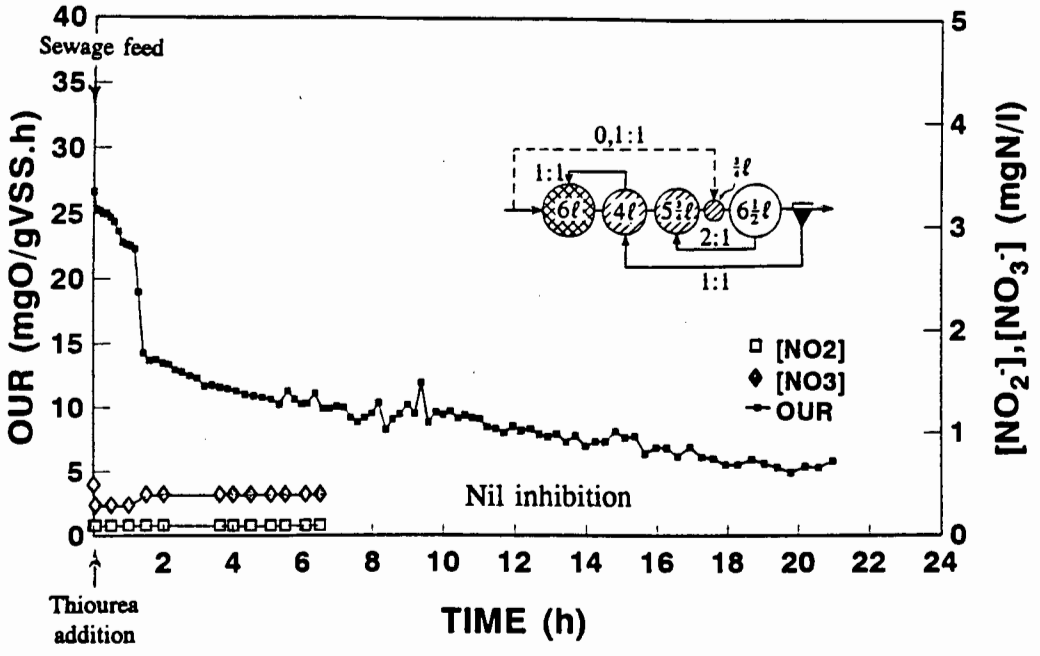

Fig 5.21: Oxygen utilization rate (OUR in mgO/gVSS.h) with time (h) for Batch Test 22 for sludge from a multireactor nitrification–denitrification biological excess phosphorus removal (NDBEPR) system with nitrite present under prior anoxic conditions.

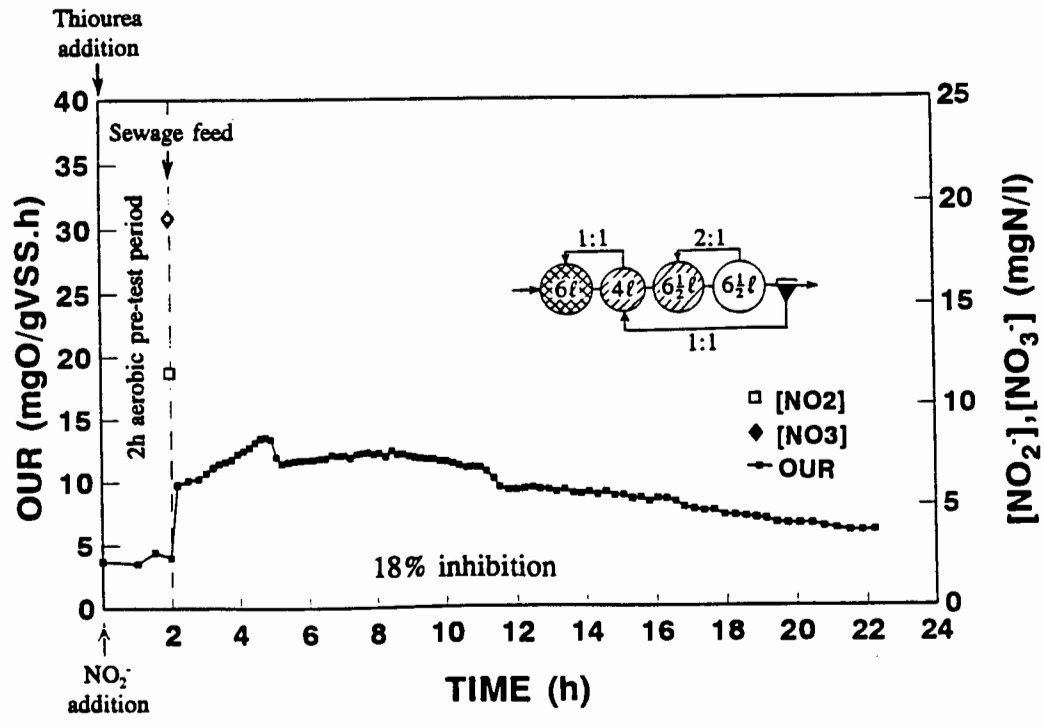

Fig 5.22: Oxygen utilization rate (OUR in mgO/gVSS.h) with time (h) for Batch Test 24 for sludge from a multireactor nitrification–denitrification biological excess phosphorus removal (NDBEPR) system with nitrite absent under prior anoxic conditions.

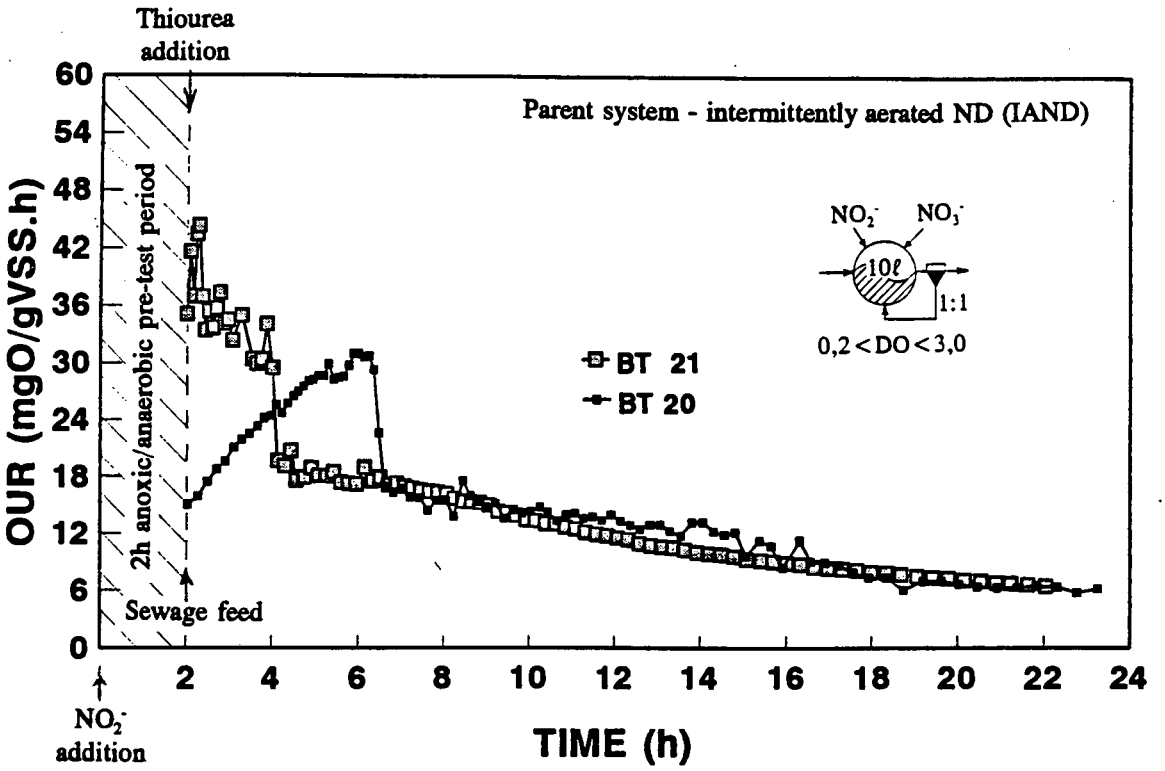

Fig 5.23: Oxygen utilization rate (OUR in mgO/gVSS.h) with time (h) for Batch Tests 20 and 21 for sludge from an intermittently aerated nitrification–denitrification (IAND) system with nitrite present (BT20) and absent (BT21) under prior anoxic conditions.

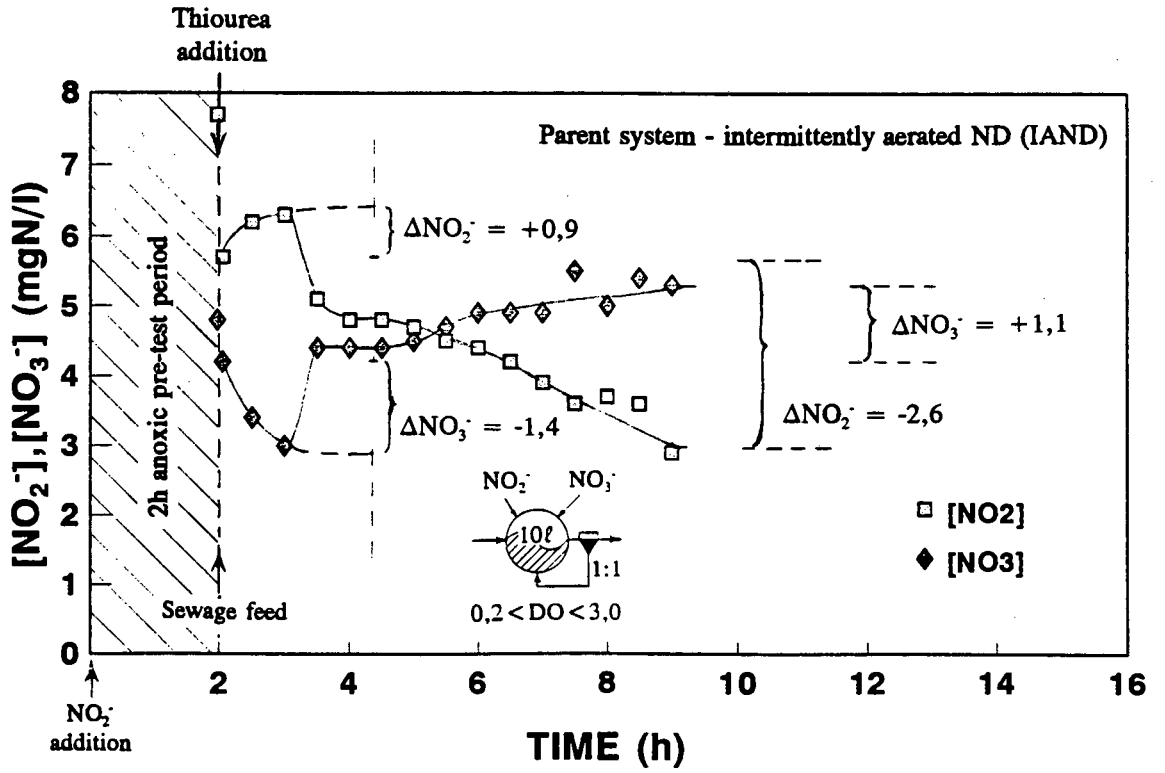

Fig 5.24: Changes in nitrate ($mgNO_3^-$–N/ℓ) and nitrite ($mgNO_2^-$–N/ℓ) concentration with time after feeding for Batch Test 20.

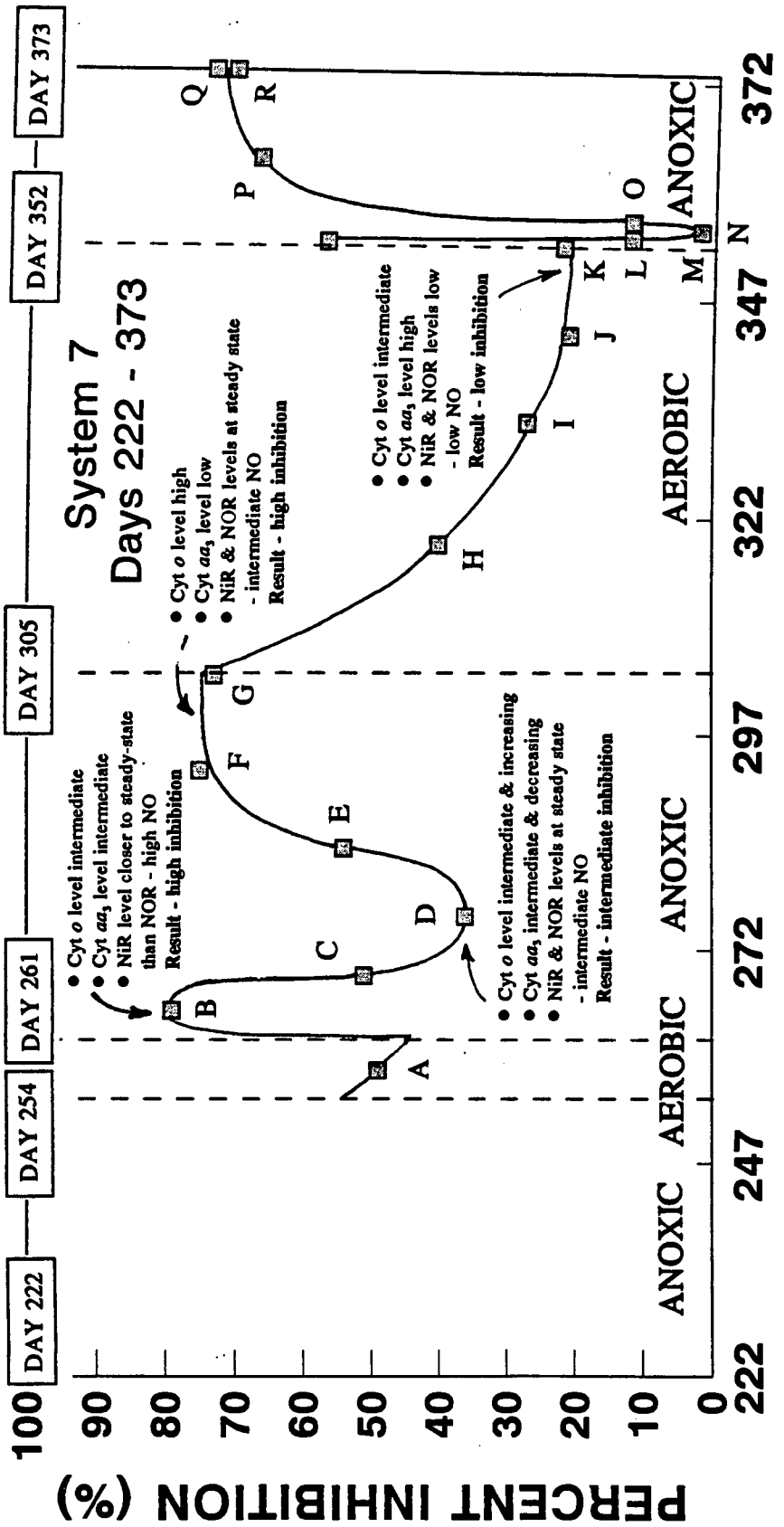

Fig. 5.25: Percentage aerobic inhibition (%) with time for sludge from System 7, resulting from changes between aerobic and anoxic conditions.

From the principles of the model, the nett increase in maximum inhibition with time results from an increase in nitrite reductase which produces nitric oxide, an increase in cytochrome *o*, and a reduction in cytochrome aa_3 synthesis, the interaction between the nitric oxide and the cytochrome *o* resulting in inhibition.

The initial rapid increase, then decrease in inhibition can be explained by examining the *rates* of synthesis of nitrite reductase and nitric oxide reductase. In modelling synthesis of the nitrogen oxide reductases after an aerobic period during which the levels of the reductases decrease, each of the reductases is synthesized in response to the presence of its reactant and at a rate proportional to the concentration of the reactant. Thus, nitrite reductase is synthesized in response to nitrite, and nitric oxide reductase is synthesized in response to nitric oxide. A change from aerobic to anoxic conditions with nitrite present, results in an initial synthesis of nitrite reductase; reduction of nitrite causes a high concentration of nitric oxide prior to synthesis of nitric oxide reductase. The period between nitrite reductase synthesis and nitric oxide reductase synthesis will result in a high nitric oxide concentration and an initial high level of inhibition due to interaction of nitric oxide with cytochrome *o*. Synthesis of nitric oxide reductase results in a reduction in nitric oxide concentration and a decrease in inhibition with time. Between Day 276 and Day 305 the level of cytochrome o increases and the level of cytochrome aa_3 decreases, during which time the level of intracellular nitric oxide is stable and inhibition increases with time.

Although the results of the experiments described above are only an indirect measure of the level of synthesis of the enzymes of the aerobic and anoxic ETP's, several aspects provide credibility for the aspects of the conceptual model describing changes in the synthesis of aerobic and anoxic respiratory ETP enzymes; the very definite trends associated with inhibition of aerobic respiration following aerobic and anoxic growth conditions, and the reproducibility of the trend in changes in inhibition associated with changes from aerobic to anoxic conditions.

- ***Exposure of aerobically grown sludge to anoxic conditions will reduce the denitrification potential of the sludge***

Anoxic denitrification batch tests (DBT) were conducted on Day 350 (DBT1) and Day 352 (DBT2) on sludge from System 7, operated under aerobic conditions , and on Day 355 (DBT3) and Day 364 (DBT4) on sludge from System 7 operated under anoxic conditions. The results of the batch tests are illustrated in Fig 5.26. For

analysis purposes, the change in nitrate concentration is divided into approximately linear sections and the progressively increasing rates of nitrate reduction in any one test are labelled $k^1_{NO_3^-}$, $k^2_{NO_3^-}$, and $k^3_{NO_3^-}$ and the rate of nitrite reduction is labelled $k^1_{NO_2^-}$. The increase in nitrate concentration during the first 20 minutes of each test is attributed to nitrate mixing problems after the addition of nitrate at the same time as addition of substrate. The variations in $k^1_{NO_3^-}$ and $k^1_{NO_2^-}$ from DBT1 to DBT4 are plotted in Fig 5.27 and are discussed separately below.

Nitrate reduction: For sludge developed under aerobic conditions, the initial rates of nitrate reduction ($k^1_{NO_3^-}$) were very low for DBT1 (1,15 mgN/gVSS.h) and DBT2 (0.88 mgN/gVSS.h).[8] The initial rate of nitrate reduction for DBT2 for the sludge subjected to aerobic conditions for a slightly longer time (2 days) than DBT1, is slightly lower than that for DBT1. This resulted from a slightly greater degree of degradation of nitrate reductase with continued exposure to aerobic conditions. On Day 352 System 7 was made anoxic, and after 3 days of anoxic conditions with nitrate present, the initial rate of nitrate reduction ($k^1_{NO_3^-}$ = 4,33 mgN/gVSS.h) was considerably higher than the value under aerobic conditions, and after a further 12 days of anoxic conditions, the initial rate of nitrate reduction ($k^1_{NO_3^-}$ = 11,68 mgN/gVSS.h) increased further. This resulted from increased synthesis of nitrate reductase with exposure to anoxic conditions, in agreement with the principles of the model.

Nitrite reduction: Figure 5.27 illustrates that similar results to those described for nitrate reduction were measured also for nitrite reduction under aerobic and anoxic conditions. The true rate of nitrite reduction ($k^1_{NO_2^-}$) was calculated by subtracting the average of the first two rates of nitrate reduction (which equals the rate of formation of NO_2^-), from the measured rate of nitrite formation during the same period. Under continuous aerobic conditions, initial rates of nitrite reduction were very low for DBT1 ($k^1_{NO_2^-}$ = 0,36 mgN/gVSS.h) and DBT2 ($k^1_{NO_2^-}$ = 0,50 mgN/gVSS.h). This resulted from decreased synthesis of nitrite reductase with time under aerobic conditions in agreement with the principles of the model. Under anoxic conditions, the initial nitrite denitrification rate increased from DBT3 ($k^1_{NO_2^-}$ = 3,10 mgN/gVSS.h) to DBT4 (10,72 mgN/gVSS.h). A noteworthy feature of the

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[8] The notation $k^1_{NO_3^-}$, $k^2_{NO_3^-}$ and $k^3_{NO_3^-}$ to indicate the initial (first), second and third rates of nitrate reduction are not to be confused with the first and second rates of nitrate reduction normally employed in wastewater research to denote nitrate reduction with RBCOD and SBCOD and endogenous matter respectively.

results is that for sludge grown under anoxic conditions and subjected to anoxic batch tests (DBT3 and DBT4), nitrite accumulated to a greater degree in DBT3 (5,0 mgNO_2^-–N/gVSS.h) than in DBT4 (1,2 mgNO_2^-–N/gVSS.h), indicating that after changing System 7 from aerobic to anoxic conditions, nitrate reductase synthesis was initially considerably more rapid than nitrite reductase synthesis. These results are in agreement with the principles of the model in which sequential formation of the nitrogen oxide reductases (in the order, nitrate–, nitrite–, nitric oxide– and nitrous oxide reductase) under anoxic conditions following steady–state aerobic conditions, results in sequential formation of the nitrogen oxides in the order, nitrate, nitrite, nitric oxide, and nitrous oxide (see Fig 5.7).

5.7 CLOSURE

In Part I of this Chapter a conceptual model was outlined for the major biochemical respiratory mechanisms involved when a heterotrophic facultative organism (representing the heterotrophic facultative mass of activated sludge) is subjected to steady–state aerobic, steady–state anoxic and changes from steady–state aerobic to anoxic and steady–state anoxic to aerobic conditions. In Part II the major proposals and implications of the model were tested with aerobic inhibition batch test procedures which examined the effect on aerobic respiration of anoxic and anaerobic pre–test conditions, nitrite and nitrate concentrations under anoxic conditions, readily and slowly biodegradable substrate, and synthesis of aerobic and denitrifying enzymes under anoxic and aerobic conditions. Anoxic batch tests examined the effect of aerobic and anoxic conditions on the denitrifying enzymes, nitrate– and nitrite reductase. The experimental results provide substantive evidence that supports the conceptual biochemical model for facultative organism respiration.

CHAPTER 6

PROLIFERATION OF AA FILAMENTOUS ORGANISMS: A BIOCHEMICAL/MICROBIOLOGICAL MODEL FOR BULKING

ABSTRACT

A model is presented that describes the competitive growth behaviour of floc–forming and AA filamentous[1] organisms in long sludge age nitrogen (N) and nitrogen and phosphorus (N & P) removal activated sludge systems. The model, referred to as the bulking model, establishes the potential for filamentous organism proliferation under various aeration and substrate feeding régimes. Based on the principles of the model, system configuration and operational procedures are proposed for the amelioration of bulking and are tested experimentally. To examine the general applicability of the bulking model it is tested through application to the results of the experiments described in Chapter 3.

6.1 INTRODUCTION

The objective of the conceptual biochemical model for aerobic facultative heterotrophic organism respiration developed in Chapter 5, was to establish a basis by which the mechanisms of respiration of the facultative organism mass in activated sludge could be understood. In this Chapter the biochemical model is applied to filamentous and floc–forming organisms, to develop a microbiological model for substrate competition by these organisms as a means of explaining the proliferation of AA filaments in N and N & P removal systems. Implicit to the formulation of the bulking model is the assumption required by the biochemical model, that both filamentous and floc–forming organisms are aerobic facultative heterotrophs.

6.2 STATEMENT OF HYPOTHESIS

In activated sludge systems, floc–formers and filaments compete for mutually growth–limiting substrate. Under completely aerobic or completely anoxic conditions, the floc–formers outcompete the filaments for substrate due to higher substrate utilization rates and filament growth is restricted. In nitrification–denitrification

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[1] Throughout description of the bulking model, the terms *filamentous* and *filaments* refer specifically to *AA filaments*, (formerly designated low F/M filaments) which are associated with poorly settling sludges in long sludge age N and N & P removal systems.

the competition for substrate under subsequent aerobic conditions and filaments gain an advantage. Given sufficient advantage, the filaments proliferate with time.

6.3 EXAMINATION OF HYPOTHESES

Steady–state anoxic conditions

Under anoxic conditions in which one or both of the ionic nitrogen oxides nitrate or nitrite are available and oxygen is absent, a major difference between the filaments and floc–formers is in their anoxic respiratory pathways, as described above.

For the floc–formers; of the oxidases, only cytochrome *o* is synthesized and cytochrome aa_3 either is absent, or present at a basal level; of the reductases, all are synthesized to their maximum level as illustrated in Fig 6.1a.

For the filaments; of the oxidases, only cytochrome *o* is synthesized, and cytochrome aa_3 either is absent or present at a basal level; of the reductases, only nitrate reductase is synthesized, as illustrated in Fig 6.1b.

Under conditions in which nitrate and nitrite are present in sufficient quantities so as not to be limiting, for the two groups of organisms the energetic yield of their respective pathways is equal. Electrons, which are transferred to nitrate–, nitrite–, nitric oxide–, or nitrous oxide reductase for the floc–formers, or to nitrate reductase for the filaments, pass two proton–pumping (energy conserving) sites, Sites I and II. Therefore, under conditions in which nitrate is not limiting, the ability to execute the denitrification pathway through each of the intermediates, does not endow the floc–formers with an energetic benefit in comparison with the filaments. Under conditions in which nitrate is limiting and substrate is in excess, the ability of floc–formers to utilize each of the sequentially produced denitrification intermediates as an electron acceptor endows them with a greater energetic benefit than the filaments (for which only nitrate can be utilized as electron acceptor). A more complete discussion of this aspect is given in Chapter 4.

In conclusion, under steady–state anoxic conditions with sufficient nitrate present, theoretically, floc–formers gain no energetic advantage over the filaments for each nitrate molecule reduced, and the low proportion of filaments which develop under steady–state anoxic conditions (see Chapter 3) is a consequence of a lower specific substrate utilization/growth rate for filaments than floc–formers.

Steady–state aerobic conditions

Under aerobic conditions with a concentration of DO greater than 2 mgO/ℓ, the filaments and floc–formers utilize oxygen as electron acceptor.[2] For floc–formers and filaments, both of the oxidases cytochrome *o* and cytochrome aa_3 are synthesized, cytochrome aa_3 to its maximum level and cytochrome *o* to a considerably reduced level ($\approx$ 30% of the level under anoxic conditions), and the reductases are synthesized at a basal level as indicated in Figs 6.2a and 6.2b for floc–formers and filaments respectively. For both floc–formers and filaments, the mechanisms of electron transfer to the complexes and finally to oxygen during respiration are the same as described in the biochemical model for facultative heterotrophs under aerobic conditions. In their transferal to oxygen the greater proportion of electrons pass to cytochrome aa_3, in the process of which three proton–pumping (energy conserving) sites, Sites I, II and III are passed. Under steady–state aerobic conditions, theoretically, floc–formers gain no energetic advantage over the filaments for each oxygen molecule reduced, and it is concluded that the low proportion of filaments which develop under steady–state aerobic conditions (see Chapter 3) results from a lower specific substrate utilization/growth rate for filaments than floc–formers.

Frequent alternation between anoxic and aerobic conditions

Level of synthesis of enzymes of the ETP

In the preceding two sections, the status of the ETP under steady–state aerobic and steady–state anoxic conditions is described. As demonstrated in Chapter 5, maximum synthesis of aerobic enzymes may take up to three sludge ages to be effected when conditions change between anoxic and aerobic conditions. However, in ND and NDBEPR systems the residence times in the anoxic and aerobic zones are in the order of hours, and with respect to the electron transferring complexes of the ETP, neither the oxidases nor the reductases will be synthesized at their maximum levels. Consequently, the ETPs of both the filaments and floc–formers are conceptualized as having all the complexes present but at *lower* levels than under steady state anoxic and aerobic conditions, irrespective of the immediate conditions to which they are exposed. The ETPs of the floc–formers and filaments

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[2]For the purposes of the model, it is assumed that the filaments and floc–formers switch from nitrate/nitrite as electron (acceptor) to oxygen as electron acceptor at about the same concentration of DO. However, equal justification could be given to the argument that as a consequence of the protrusion of the filaments from the floc into the bulk liquid, they react to a change in DO more rapidly than the floc–formers (oxygen at low concentrations may not penetrate into the floc).

are illustrated in Figs 6.3a and 6.3b respectively for conditions under which filaments proliferate, i.e. 30 to 40 percent aerobic. The complexes are present at a level intermediate between the levels under steady–state aerobic and steady–state anoxic conditions, depending on the proportion of exposure of the organisms to aerobic and anoxic conditions. For the denitrifying enzymes, the level of each of the nitrogen oxide reductases increases with time under anoxic conditions and decreases with time under aerobic conditions. For the oxidases under anoxic conditions, cytochrome aa_3 decreases and cytochrome *o* increases, and under aerobic conditions, cytochrome aa_3 increases and cytochrome *o* decreases.

Activity of enzymes of the ETP

Given that the level of the enzymes does not vary considerably during exposure to relatively short (hours) alternating anoxic–aerobic conditions, low or negligible rates of nitrogen oxide reduction under aerobic conditions are a consequence of inactivation of the reductases by oxygen. Activation of the reductases occurs in the absence of, or at low concentrations of oxygen. This applies to both filaments and floc–formers.

Biodegradability of substrate available under anoxic and aerobic conditions

For long sludge age ND and NDBEPR systems fed normal municipal substrate, by far the majority of substrate available under aerobic and anoxic conditions is slowly biodegradable COD (SBCOD). As noted in Chapter 5, this has implications with respect to intracellular accumulation of nitric oxide generated by denitrification of nitrite under anoxic and aerobic conditions. Nitric oxide does not accumulate through denitrification with readily biodegradable substrate under anoxic or aerobic conditions but does accumulate through denitrification with slowly biodegradable substrate under both conditions.

Inhibition of floc–former substrate utilization under aerobic conditions

Of particular interest to this model is a mechanism described at length in Chapter 5; when facultative organisms are subjected to a change from anoxic to aerobic conditions with nitrite present, they are inhibited in their utilization of substrate by accumulated intracellular nitric oxide. It is not the intention to again describe in detail the biochemical mechanisms which contribute to inhibition, but rather to indicate the applicability of the mechanisms of inhibition of substrate utilization under aerobic conditions to filamentous and floc–forming organisms.

The mechanism for inhibition of substrate utilization under aerobic conditions by nitric oxide is applicable to floc–formers, but not to filaments. This is a consequence of the postulate that under anoxic conditions which precede aerobic conditions, floc–formers denitrify nitrate to the end–product dinitrogen through each of the intracellular denitrification intermediates, nitrite, nitric oxide, and nitrous oxide, with nitric oxide accumulating with utilization of slowly biodegradable substrate. Under subsequent aerobic conditions, intracellular nitric oxide inhibits aerobic respiration. In the biochemical model, facultative organisms inhibited in aerobic respiration transfer electrons to the denitrification pathway and denitrify nitrite under aerobic conditions. (As indicated in the biochemical model, nitrate is not denitrified under aerobic conditions due to impermeability of the cytoplasmic membrane to nitrate, preventing access to its reductase). Similarly, floc–formers inhibited in aerobic respiration denitrify nitrite via the same mechanisms, with an accompanying reduction in substrate utilization rate and energetic yield with nitrite as electron acceptor to that with oxygen as electron acceptor. In contrast to floc–formers, under the same conditions, filaments reduce nitrate to the end–product nitrite, and do not produce intracellular nitric oxide. Consequently, under subsequent aerobic conditions, aerobic respiration in filaments is not inhibited providing them with an advantage in competition with the floc–formers for substrate under aerobic conditions.

As described in the biochemical model for facultative respiration, a requirement for inhibition of aerobic respiration is the presence of intracellular nitric oxide during a change from anoxic to aerobic conditions. *However intracellular nitric oxide cannot be directly or easily monitored; since nitrite is the precursor to nitric oxide in the denitrification pathway, in the bulking model, nitrite is used as an indicator of the presence of nitric oxide.* In the absence of nitrite, during a change from anoxic to aerobic conditions, floc–formers are not inhibited and compete for substrate, such that filaments gain no advantage under aerobic conditions. The biochemical interactions which take place during a change to aerobic conditions from anoxic conditions in which nitrite is present or absent are described extensively in the biochemical model for facultative organism respiration and apply directly to floc–formers. A requirement to maintain inhibition under aerobic conditions is the presence of nitrite in the aerobic zone and the conditions which cause nitrite to be present at concentrations sufficient to induce inhibition of respiration and to maintain inhibition are described below.

Concerning the source of nitrite under aerobic conditions, it is well established that under steady–state aerobic conditions in which ammonium is not limiting, the rate of nitrification of nitrite to nitrate by *Nitrobacter* is somewhat faster than the rate of nitrification of ammonium to nitrite by *Nitrosomonas* and nitrite does not accumulate. However, a finding of the review of nitrification in Appendix E is that unaerated (anoxic or anaerobic) conditions inactivate the two groups of nitrifying organisms *Nitrosomonas* and *Nitrobacter* such that their rates of oxidation are reduced under subsequent aerobic conditions, and of the two groups, *Nitrobacter* are more sensitive to low concentrations of DO or the absence of DO than *Nitrosomonas.* As a consequence, nitrite is not nitrified to nitrate at the same rate as it is produced and it accumulates under each successive aerobic condition of the alternating anoxic–aerobic conditions.

Conceivably, both production of nitrite under aerobic conditions, and production of nitrite under anoxic conditions can contribute to the pool of extracellular nitrite in systems with alternating anoxic–aerobic conditions, and it is unclear as to which source is the cause of high concentrations of nitrite frequently observed in these systems. However given the significant intolerance of the nitrifying organisms to changes in other environmental conditions such as temperature, pH, and toxins, it would seem likely that the nitrifying organisms, more so than the denitrifying organisms are responsible for high nitrite concentrations.

6.4 MODEL EVALUATION AND EXPERIMENTAL TESTING

In evaluating the model, two avenues of investigation are employed; (1) the implications of the model are experimentally tested to determine the general validity of the principles of the model, (2) the model is tested against experimental observations made during the exploratory phase of the experimental programme to determine its general applicability.

(1) Examination of model implications

A difference in the relative extents of reduction of nitrate by floc–formers and filaments

If floc–formers denitrify nitrate through each of the denitrification intermediates to the end–product dinitrogen, and filaments reduce nitrate to the end–product nitrite, then under anoxic conditions a sludge containing a high proportion of filaments (high DSVI) should accumulate nitrite, but a sludge containing a high proportion of floc–formers (low DSVI) should produce greater quantities of dinitrogen.

Table 6.1: Results of nitrate reduction tests conducted on a sludge sample from a continuous anoxic system (System 7, Day 255) with a low DSVI, and an intermittent aeration system (System 9, Day 228) with high DSVI.

NITRATE REDUCTION TESTS					
System 7 Continuous anoxic Day 255	NO_2^-	$N_2(g)$	System 9 MLE 70% unaerated Day 228	NO_2^-	$N_2(g)$
1	-	+	1	+	-
2	+(weak)	+(weak)	2	+	-
3	-	+	3	+	-
4	-	+	4	+	-
5	-	+	5	+	-
6	-	+	6	+	-
7	-	+	7	-	+
8	-	+	8	+	-
9	+	-	9	+	-
10	+	-	10	-	+

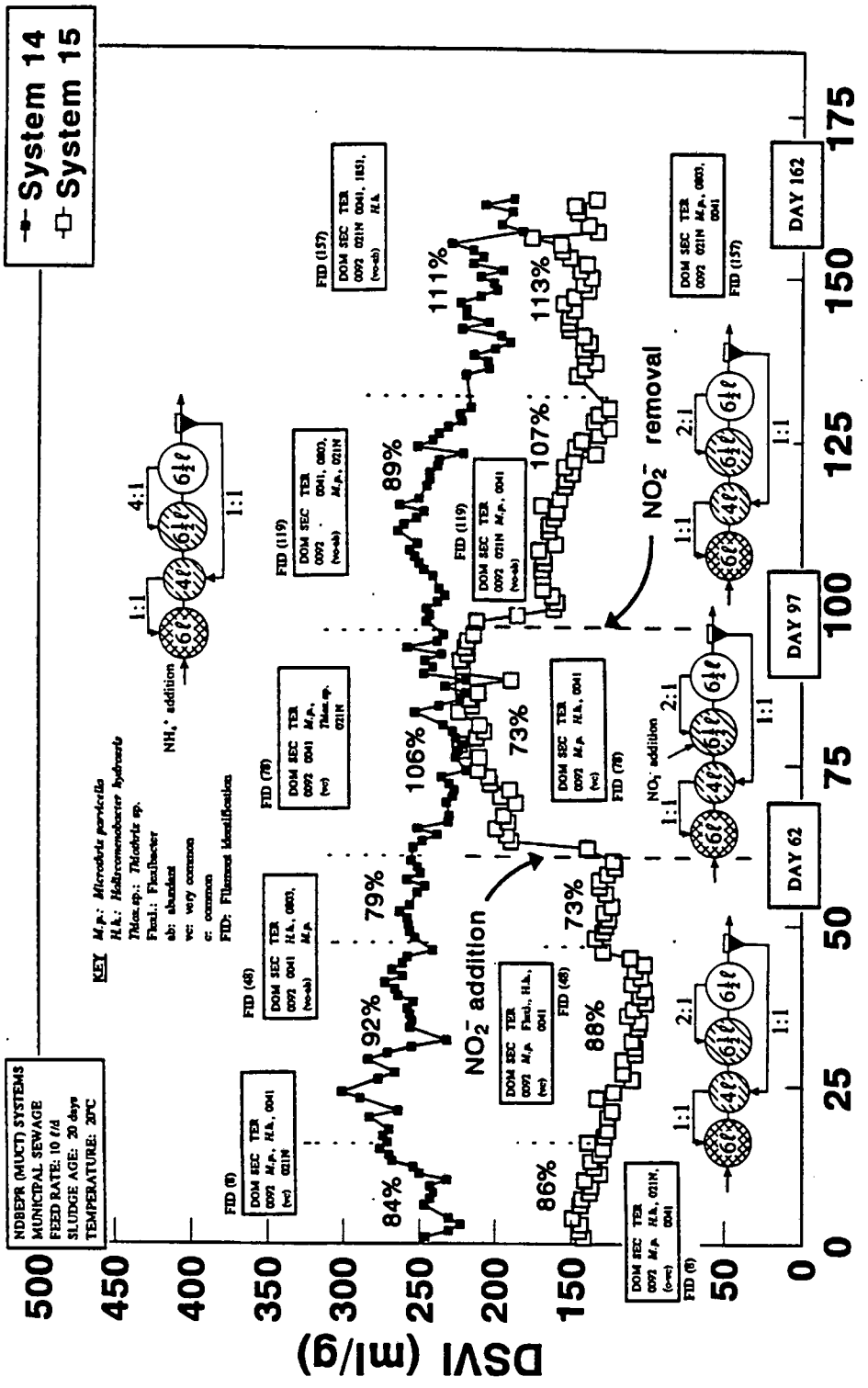


Fig 6.4: Sludge settleability as dilute sludge volume index (DSVI in mℓ/g) with time (h) for System 14 (NH_4^+ addition to influent) and System 15 (NO_2^- addition to 2nd anoxic reactor).

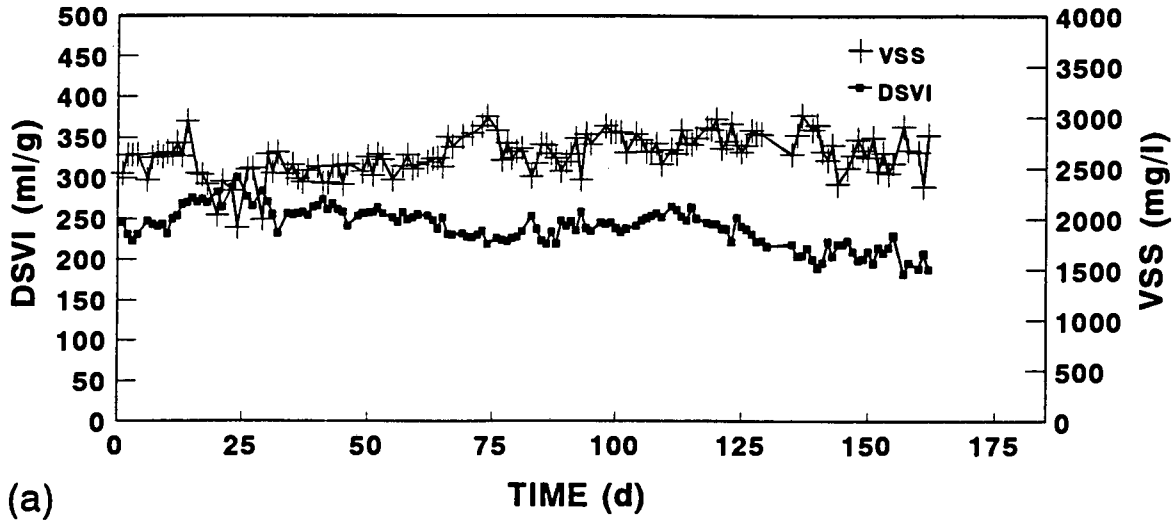
gain an increasing advantage in respiring under aerobic conditions because oxygen is not limiting. This allows filaments to proliferate to a greater degree at increasingly higher DO concentrations in the aerobic zone of intermittently aerated systems.

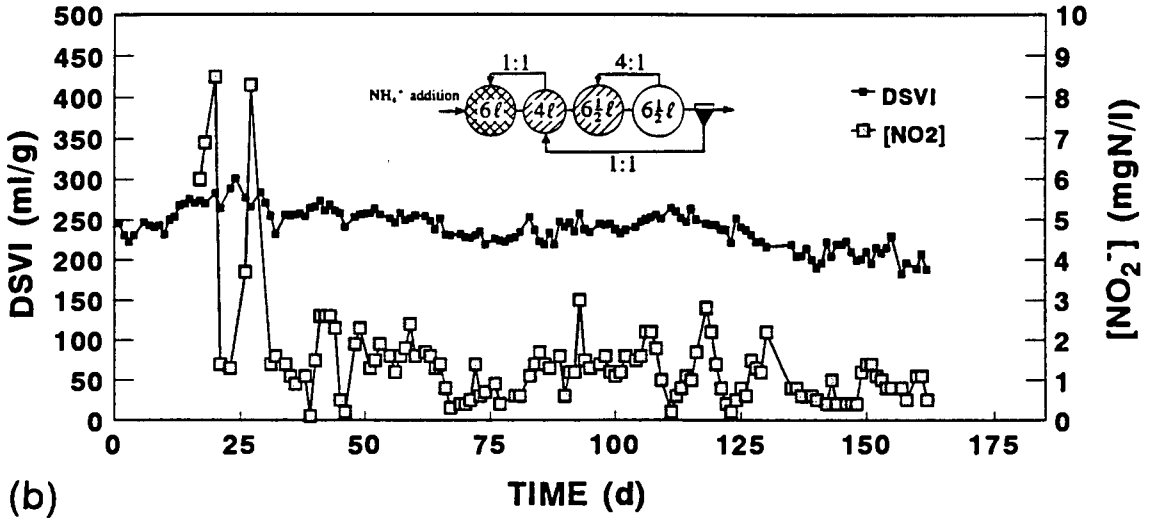
Substrate biodegradability

(i) *RBCOD fed throughout the intermittent aeration sequence resulted in high DSVI values (System 7, Days 63 to 94; System 8, Days 95 to 127):* This result was unexpected since the rate of supply of electrons from RBCOD should be sufficient to remove any accumulated intracellular nitric oxide. When RBCOD was fed to System 8 the DSVI initially decreased rapidly as the growth of the AA filament *M. parvicella* was reduced, but the DSVI then increased due to *H. hydrossis*, a filament categorized as low DO, a category of filaments to which the model does not apply.

(ii) *SBCOD fed throughout the intermittent aeration sequence resulted in high DSVI values (System 7, Days 95 to 127, System 8, Days 64 to 94):* Under conditions of high nitrite concentration entering the aerobic zone, a low rate of supply of electrons from SBCOD would be insufficient to remove the intracellular accumulation and inhibitory effect of nitric oxide.

(iii) *RBCOD fed during the anoxic zone of the intermittent aeration sequence resulted in high DSVI values (System 7, Days 128 to 162), but RBCOD fed during the aerobic zone of the intermittent aeration sequence resulted in low DSVI values (System 8, Days 163 to 192):* For RBCOD fed during the anoxic zone only, in the aerobic zone, where the concentration of nitrite was > 1,0 mgN/ℓ, SBCOD was the only available substrate, and although the floc–formers were not inhibited upon entering the aerobic zone, the low rate of supply of electrons from SBCOD under aerobic conditions with nitrite present induced inhibition and a high DSVI resulted. For RBCOD fed during the aerobic zone only, with the high nitrite concentration (> 1,0 mgN/ℓ) in the anoxic zone with only SBCOD available, the floc–formers are inhibited. Upon entering the aerobic zone with RBCOD present, irrespective of the concentration of nitrite present, floc–former inhibition is relieved and they out–compete the filaments for substrate.





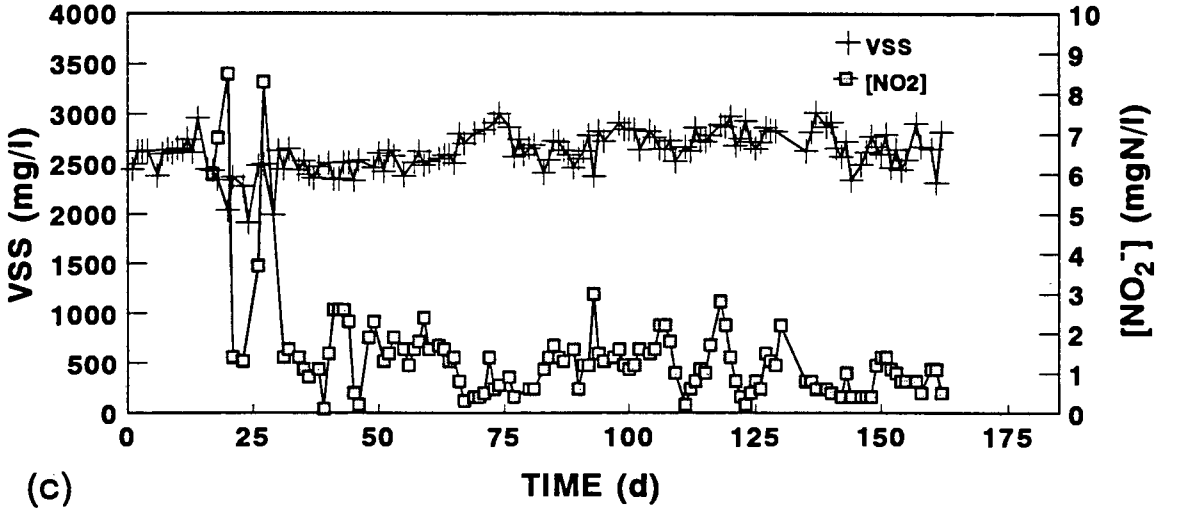


Fig 6.6: (a) DSVI and VSS with time, (b) DSVI and 2nd anoxic reactor NO_2^- concentration with time, and (c) VSS and 2nd anoxic reactor NO_2^- concentration with time for System 14.

intermittent aeration) the COD mass balances were low, and for systems with low DSVIs (produced through continuous aeration) the COD mass balances were high. In summary, combining the results of multi–reactor systems (Systems 12 to 15) and single reactor systems (Systems 5 to 8) it can be concluded that: In systems in which high DSVI values develop (many filaments) (i.e. systems in which sludge is exposed to alternating anoxic–aerobic conditions), both the COD and nitrogen mass balances are low, and in systems in which low DSVI values develop (few filaments) (i.e. continuous aerobic systems), both the COD and nitrogen mass balances are high.

Concerning the relationship between VSS and nitrite concentration, interaction of nitric oxide with cytochrome oxidase results in redirection of electrons to nitrite reductase, and the resultant aerobic denitrification yields only two–thirds the energy of uninhibited aerobic respiration. The consequence is that production of sludge mass is reduced. Concerning the relationship between the high DSVIs produced by high nitrite concentrations, and the low COD and nitrogen mass balances which accompany high DSVIs, a consequence of aerobic denitrification of nitrite by floc–formers is that nitrogen and COD removed under aerobic conditions will be unaccounted for in the nitrogen and COD mass balances. Thus during periods of high or increasing DSVI, low nitrogen and COD mass balances are measured.

In summary, for sludge subjected to alternating anoxic–aerobic conditions with nitrite present, floc–former aerobic respiration is inhibited by interaction of nitric oxide with cytochrome oxidase, filaments proliferate, and the DSVI increases. Electrons (originating from COD) are then directed to nitrite reductase and under aerobic conditions nitrite is reduced. Because of the lower yield of respiration with nitrite, compared to respiration with oxygen, the VSS of the sludge is less than under conditions in which inhibition of floc–former aerobic respiration is not induced (i.e. under continuous aerobic conditions, or under alternating anoxic–aerobic conditions in which nitrite is absent). Under conditions in which floc–formers are not inhibited, electrons are not redirected to nitrite reductase and nitrite is not utilized under aerobic conditions, and the nitrogen and COD balances are high.

6.5 CONTROL PROCEDURES FOR AA FILAMENT BULKING

In summary, experimental examination of the implications of the model and application of the model to a range of experimental results provide support for the validity of the model. To assist in development of procedures for control of filament

is to design the anoxic mass fraction/a– (aerobic–anoxic) recycle of the system such that the denitrification potential of the system is greater than the mass of NO_3^- and NO_2^- recycled to it, i.e. complete denitrification in the anoxic zones is achieved. This can be accomplished by; (i) increasing the unaerated mass fraction, or, (ii) reducing the a–recycle.

In the event that inflexible system configurations do not allow for reduction of the a–recycle, or unusual influent wastewater characteristics do not practically allow for removal of all NO_3^- and NO_2^- recycled to the anoxic reactor or zone, attention is directed not at the extracellular NO_2^-, but at intracellular NO, and provisions are made for its reduction under both aerobic and anoxic conditions. This can be accomplished by:

(i) Inclusion of a small aerobic reactor immediately upstream of the main aerobic reactor to which a fraction of the influent sewage is fed. From the biochemical model, intracellular NO is reduced rapidly under aerobic conditions with RBCOD and inhibition is not induced. Experiments with aerobic batch tests (see Chapter 5) in which inhibition is relieved in the presence of sufficient quantities of RBCOD offer support for this proposal which is currently under investigation but which is not described here.

(ii) Inclusion of a small anoxic reactor immediately upstream of the main aerobic reactor to which a fraction of the influent sewage is fed – from the biochemical model, intracellular NO is reduced rapidly under anoxic conditions with RBCOD and inhibition is not induced under subsequent aerobic conditions. This proposal has been investigated and the experiments are described below.

A denitrifying anoxic (DENOX) reactor comprising 4 percent of the total sludge mass of MUCT System 17 was added between the 2nd anoxic reactor and the aerobic reactor, and 10 percent of the influent feed was introduced into the DENOX reactor. The MUCT + DENOX configuration and changes in DSVI with time are illustrated in Fig 6.8. Between days 50 and 76, the DSVI decreased from > 150 mℓ/g to < 100 mℓ/g. During the same period, the DSVI of a control system, System 16, with ammonium added to the influent, increased from 160 mℓ/g to > 220 mℓ/g. Following removal of the DENOX reactor from System 17 and addition of the DENOX to System 16, the DSVIs of the two systems initially acted in

accordance with expectation; the DSVI of System 17, now without the DENOX reactor, increased from < 100 mℓ/g to ≈ 170 mℓ/g in 20 days (up to day 97) and the DSVI of the System 16, incorporating the DENOX reactor, decreased from > 220 mℓ/g to ≈ 110 mℓ/g in 20 days. On Day 97 the DSVIs of the systems began to deviate from the expected behaviour. The DSVI of the DENOX system (System 16) increased to between 150 and 170 mℓ/g and the DSVI of the system without the DENOX reactor (System 17) decreased to ≈ 100 mℓ/g. At present, the causes of this deviant behaviour are unclear. However, from the experimental results obtained thus far, introduction of a small unaerated reactor between the anoxic and aerobic zones and addition of substrate containing an adequate supply of RBCOD to the small reactor appears to hold promise as a strategy for control of AA filaments.

As an aside: it should be noted that during testing of the DENOX reactor control procedure, relationships between the parameter DSVI, VSS, and nitrite concentration in the second anoxic reactor were the same as were found throughout the experimental programme; generally DSVI increased and VSS decreased with increase in nitrite concentration and DSVI decreased and VSS increased with decrease in nitrite concentration as illustrated in Fig 6.9 and Fig 6.10 for Systems 16 and 17 respectively.

6.6 CLOSURE

A wide–ranging experimental program (Chapter 3) investigating at laboratory–scale the problem of filamentous organism bulking (formerly low F/M bulking – now renamed AA bulking), resulted in the development of a biochemical model describing the fundamental biochemical mechanisms of facultative heterotrophic organisms (Chapter 5). Application of the biochemical model for facultative organism respiration to filamentous and floc–forming facultative organisms provided the means to develop a conceptual model that explains AA filamentous organism proliferation or non–proliferation. The model was evaluated against experimental data and shown to provide an explanation for observations relating to AA filament bulking. From the conceptual model, proposals for measures by which the bulking problem can be reduced or eliminated were identified and examined. Initial testing of the proposals produced encouraging results.

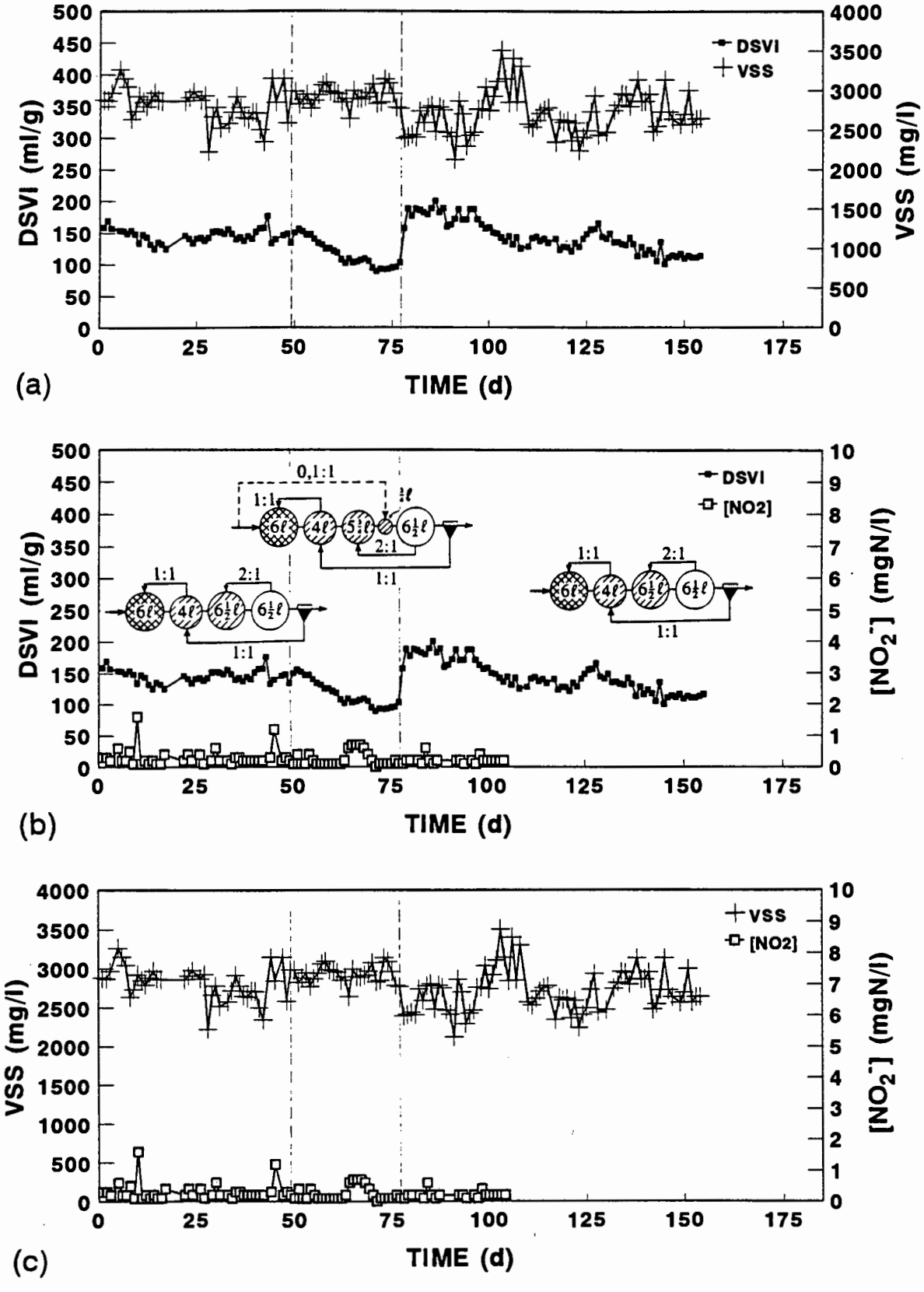


Fig 6.10: (a) DSVI and VSS with time, (b) DSVI and 2nd anoxic reactor NO_2^- concentration with time, and (c) VSS and 2nd anoxic reactor NO_2^- concentration with time for System 17.

CHAPTER 7

CONCLUSIONS AND RECOMMENDATIONS

The objective of the investigation was to determine the fundamental causes for the proliferation of low F/M filamentous organisms in long sludge age, nitrogen (N) and nutrient (N & P) removal systems.

In meeting this objective the investigation evolved in 4 sequential stages; viz., (1) a review of bulking problems and practices world-wide, (2) an exploratory experimental investigation to examine the effect of numerous factors on filament proliferation, (3) formulation and experimental testing of a conceptual biochemical model for facultative heterotrophic organism respiration, and (4) formulation and experimental testing of a conceptual microbiological model for proliferation of filamentous organisms.

(1) <u>Review of bulking</u>

- Procedures for specific control of filamentous organism bulking have suffered from lack of a single broadly applicable approach. Where procedures have been successful in specific cases, for example, with incorporation of selector reactors, application of the same procedures to other situations has often proven unsuccessful.

- The filamentous organism classification procedure of Jenkins *et al.*, (1984a) in identifying conditions giving rise to proliferation of specific organisms, has brought a measure of order to the field. However, the procedure has also proven somewhat inadequate in that filaments often proliferate under conditions not assigned to their classification. For example, in long sludge age systems, which support low F/M conditions, filaments not classified as low F/M often proliferate. Additionally, the procedure does not describe the fundamental biochemical and microbiological mechanisms which allow filaments to find a niche in activated sludge.

(2) <u>Exploratory experimental investigation</u>

- As a starting point to the experimental investigation, the use of a defined

cytochrome *o* is sparse, and more biochemical research is necessary to establish, (i) rates of synthesis and degradation of the reductases and oxidases under anoxic and aerobic conditions; (ii) placement of the reductases and oxidases with respect to the cytoplasmic membrane; (iii) mechanisms of nitrogen oxide transport to and from the reductases; (iv) mechanisms of nitrogen oxide reduction, and (v) mechanisms of interaction between nitric oxide and cytochromes *o* and aa_3.

- A method was described and tested by which indirect measurement could be made of the levels of synthesis of both the reductases and oxidases (aerobic inhibition batch tests) and the reductases only (anoxic denitrification batch tests) of facultative organisms in activated sludge subjected to changes between aerobic and anoxic conditions. The examination of this aspect was conducted on a cursory level only, and a more rigorous investigation is required, focusing in particular on the sequential production and removal of the denitrification intermediates produced by the reductases during changes between aerobic and anoxic conditions.

- The aerobic inhibition batch test method is a useful tool for determining the *potential* for filamentous organism growth via the *potential* for inhibition of floc–forming organisms through indication of the levels of their oxidases and reductases. However, filamentous organisms will proliferate in the parent system only in the presence of certain other conditions, (e.g. nitrite present prior to aerobic conditions and readily biodegradable substrate absent or at very low concentrations under aerobic conditions).

- A rapid analytical procedure is required to determine the level of aerobic and denitrifying enzymes in the mixed culture organisms of activated sludge (as opposed to procedures applicable to pure culture organisms) so as to establish the potential for inhibition of aerobic respiration and concomitant filament growth which results in bulking.

(4) <u>Microbiological/Biochemical (Bulking) model</u>

- The bulking model can explain the proliferation or not of AA filamentous organisms under essentially all the conditions to which sludge is subjected in nitrogen and nutrient removal activated sludge systems.

filamentous organisms, which can be used in the design and operation of full–scale wastewater treatment facilities.

(5) General

- Historically, the philosophy governing attempts to control filament growth has been to *increase the growth rate of floc–formers* above that of filaments for the substrate concentrations found in activated sludge, through induction of the selector effect. In contrast the results of this investigation indicate that a more appropriate philosophy is to *remove the disadvantage to floc–former growth* which results from inhibition of aerobic respiration.

APPENDIX A

DEVELOPMENT OF AND EXPERIMENTS WITH A DEFINED ARTIFICIAL SUBSTRATE

Appendix A describes the motivation behind the development and use of a defined artificial substrate. The defined substrate was used in Part I of the experimental investigation described in Chapter 3. The results of all the experimental work including daily experimental data, aerobic batch tests, COD and Nitrogen mass balances, substrate testing, and filament identifications are described herein.

A.1 BACKGROUND TO SUBSTRATE DEVELOPMENT

In the initial investigations conducted by Gabb (1988) into filamentous organism bulking in laboratory–scale nitrogen and nutrient removal systems fed municipal sewage, a problem encountered was that for each batch of sewage obtained from Mitchell's Plain full–scale sewage treatment plant (Cape Town) (approximately every 2 weeks), the initial response of the laboratory system differed.

In order to eliminate the uncertainty associated with changes in filamentous organism growth resulting from variations in municipal sewage composition, Gabb (1988) motivated the development of a defined artificial substrate as a substitute for municipal sewage as influent to laboratory systems. Additionally, the flexibility of the defined artificial substrate composition is advantageous for work concerned with the utilization of specific fractions of sewage by organisms, in which the effect of various components of the substrate on filamentous organism proliferation can be examined through their addition to and removal from the substrate.

In development of the substrate, three key objectives were set; that the artificial substrate would have; (i) a similar chemical composition to municipal sewage, (ii) a similar kinetic response, and (iii) similar filamentous organism types and bulking behaviour as the laboratory–scale units being fed municipal sewage.

A similar chemical composition refers to the classification of compounds as either soluble or particulate, as well as the correct proportions of essential nutrients such as carbon, nitrogen and phosphorus and other nutrients such as potassium, magnesium, zinc and manganese. The kinetic response of the sludge to the substrate is dependent on having the correct proportions of readily biodegradable and slowly biodegradable material as well as the correct biodegradability of both fractions, and can be determined by the oxygen utilization rate (OUR) of the sludge under specific aerobic batch test conditions. The filamentous organism types which developed in the systems fed defined substrate were identified microscopically and compared to known filament types which develop at full–scale and laboratory–scale on municipal sewage. Methods for filamentous organism identification followed the procedure of Eikelboom (1975, 1977) and Jenkins *et al.* (1984a).

The investigation was conducted in three steps:

procedure is that the maximum specific OUR is not a good parameter for assessment of the proportions of readily and slowly biodegradable COD present in the substrate because other factors affect this parameter to a considerable degree; the method of feeding (whether intermittent or continuous) (Still *et al.*, 1986) and the system configuration (Chudoba *et al.*, 1973b). In the procedure described by Ekama *et al.* (1986) for aerobic batch tests, the proportions of RBCOD and SBCOD in a substrate are determined by comparing the areas under specific parts of the OUR profile. Thus, although the two systems tested by Gabb (1988) were operated under the same conditions (i.e. same influent COD system volume, feed pattern, temperature, etc.) the method in which the response of organisms to RBCOD was determined by comparing the maximum specific OUR rather than the area under the OUR curve is not a good means of comparing the values of RBCOD contained in municipal and defined substrate. A better method for determination of readily and slowly biodegradable fractions in substrates is the "flow through activated sludge system" method (Ekama *et al.*, 1986). The results of the application of this method to the defined substrate are described in Section A.4.

(3) <u>Bacterial population development</u>

In order to apply at full scale, the results of laboratory research, it is important that the filamentous organisms developed at laboratory–scale on either municipal sewage or artificial substrate are similar to those found in full–scale systems. Gabb (1988) operated 2 single reactor batch fed long sludge age systems with a 24 hr aeration pattern of 6 hrs unaerated, 16 hrs aerated and 2 hrs for sludge settling and liquor decanting. In previous studies (Gabb et al., 1989a) laboratory–scale systems fed municipal sewage and operated with this aeration pattern developed similar filamentous organism types to those found in full–scale systems. The two laboratory–scale systems developed sludges with similar filament types and with DSVI values greater than 150 mℓ/g for longer than 3 sludge ages.

Gabb (1988) concluded that these results indicated that the competence of the defined substrate to substitute as a laboratory wastewater had been adequately demonstrated, the substrate having satisfied the three criteria outlined above, and work with the defined substrate was continued by that worker. The defined substrate composition fed to the intermittently aerated

Table A.1: Constituents of the suspensions of the defined artificial substrate.

CONSTITUENTS OF THE SUSPENSIONS OF DEFINED SUBSTRATE					
RBCOD	**Suspension concentration (g/5ℓ)**	**Vitamins**	**Suspension concentration (g/5ℓ)**	**Fats & Oils**	**Suspension concentration (g/10ℓ)**
Lactose	3,3	Pantothenic Acid	1,400	Sodium Oleate	19,5
Sodium acetate	13,8	Nicotinic Acid	1,400	Stearic acid	4,5
Sodium succinate	8,7	Biotin (D-7)	0,07	Palmitic acid	11,38
Tri-sodium citrate	26,4	Cyanocobalamin (Vit.B-12)	0,07	Tween 20	1,88
D-Glucose	3,3	Folic Acid	0,07	Tween 40	6,0
Maltose	3,3	Pyridoxine (alhydrochloride)	1,400	Tween 60	3,75
Glycerol	5,4	Cocarboxylate	1,400	Tween 80	12,0
	(mℓ/5ℓ)	p-Aminobenzoic acid	1,400	Mineral Oil	3,75
Lactic Acid	20,0	Inositol-(meso)	1,400		
Ethanol	9,0	Thiaminium dichloride	1,400		
Butanol	4,5	Riboflavin	1,400		
		Choline Chloride	1,400		
Micro-inorganic nutrients 2	**Suspension concentration (g/5ℓ)**	**Macro-inorganic nutrients 1**	**Suspension concentration (g/15ℓ)**	**Micro-inorganic nutrients 1**	**Suspension concentration (g/5ℓ)**
$NiCl_2.6H_2O$	0,250	NH_4Cl	162,0	$FeSO_4.7H_2O$	8,62
$AlCl_3.6H_2O$	0,100	K_2HPO_4	57,0	$ZnSO_4.7H_2O$	2,46
NH_4VO_3	0,025	KH_2PO_4	3,0	$MnSO_4.4H_2O$	2,46
Na_2SeO_3	0,010	$MgCl_2.6H_2O$	198,0	$CuSO_4.5H_2O$	0,50
TiO_2	0,040	$CaCl_2.2H_2O$	49,5	$CoCl_2.6H_2O$	0,50
$Na_2WO_4.2H_2O$	0,015			$Na_2MoO_4.2H_2O$	0,25
				H_3BO_3	0,50
				KI	0,12
Macro-inorganic nutrients 2	**Suspension concentration (g/15ℓ)**	**Complex Carbohydrates**	**Suspension concentration (g/15ℓ)**	**Organic Nitrogen**	**Suspension concentration (g/8ℓ)**
K_2HPO_4	57,0	Starch	50,55	Casein	10,80
KH_2PO_4	3,0	Cellulose	39,60	Peptone	20,20
$MgCl_2.6H_2O$	198,0	Agar	7,95	Yeast Extract	20,20
$CaCl_2.2H_2O$	49,5	Dextrin	69,15	Gelatin	15,80

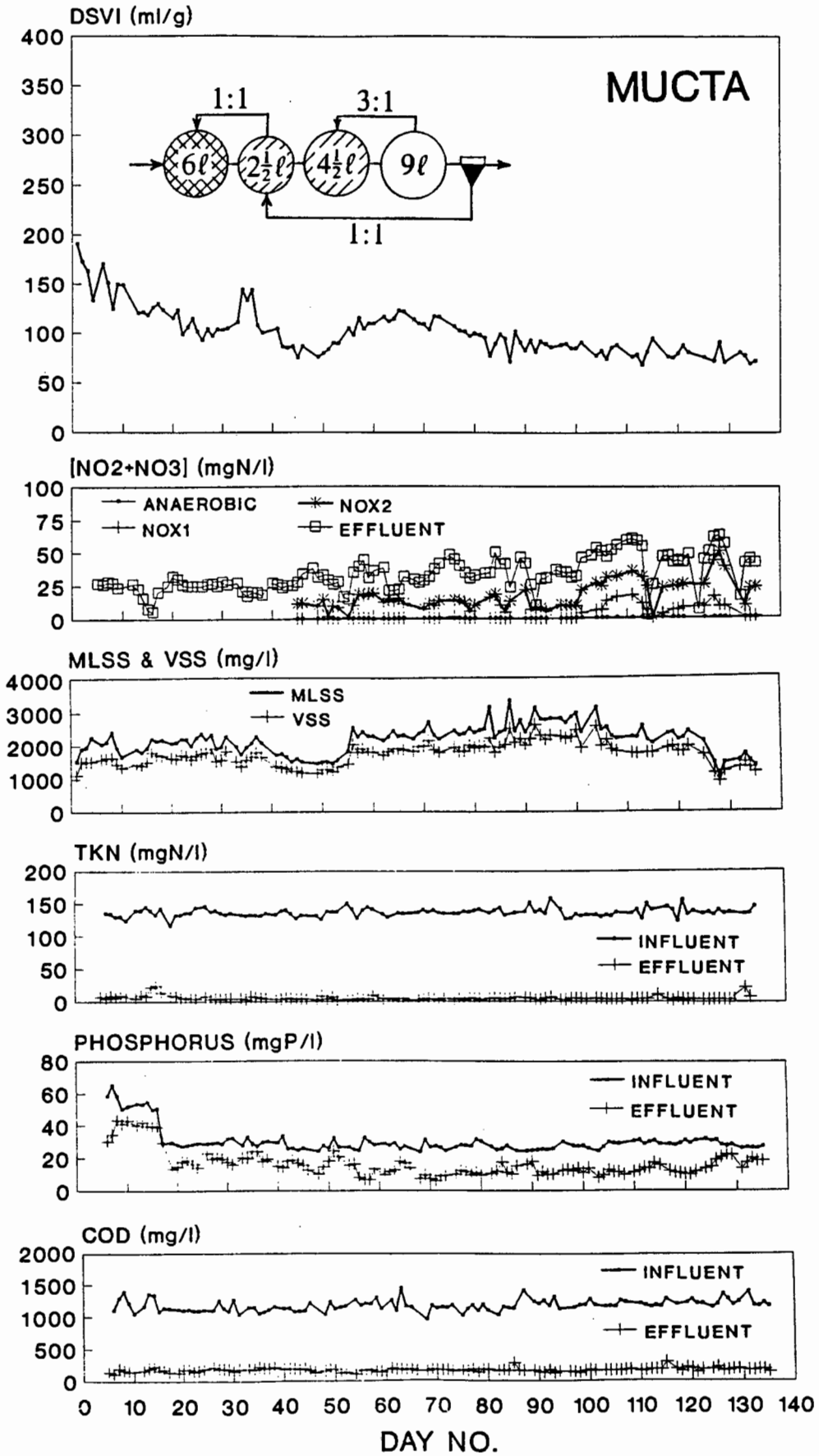


Fig A.1: Daily measured data for System MUCTA.

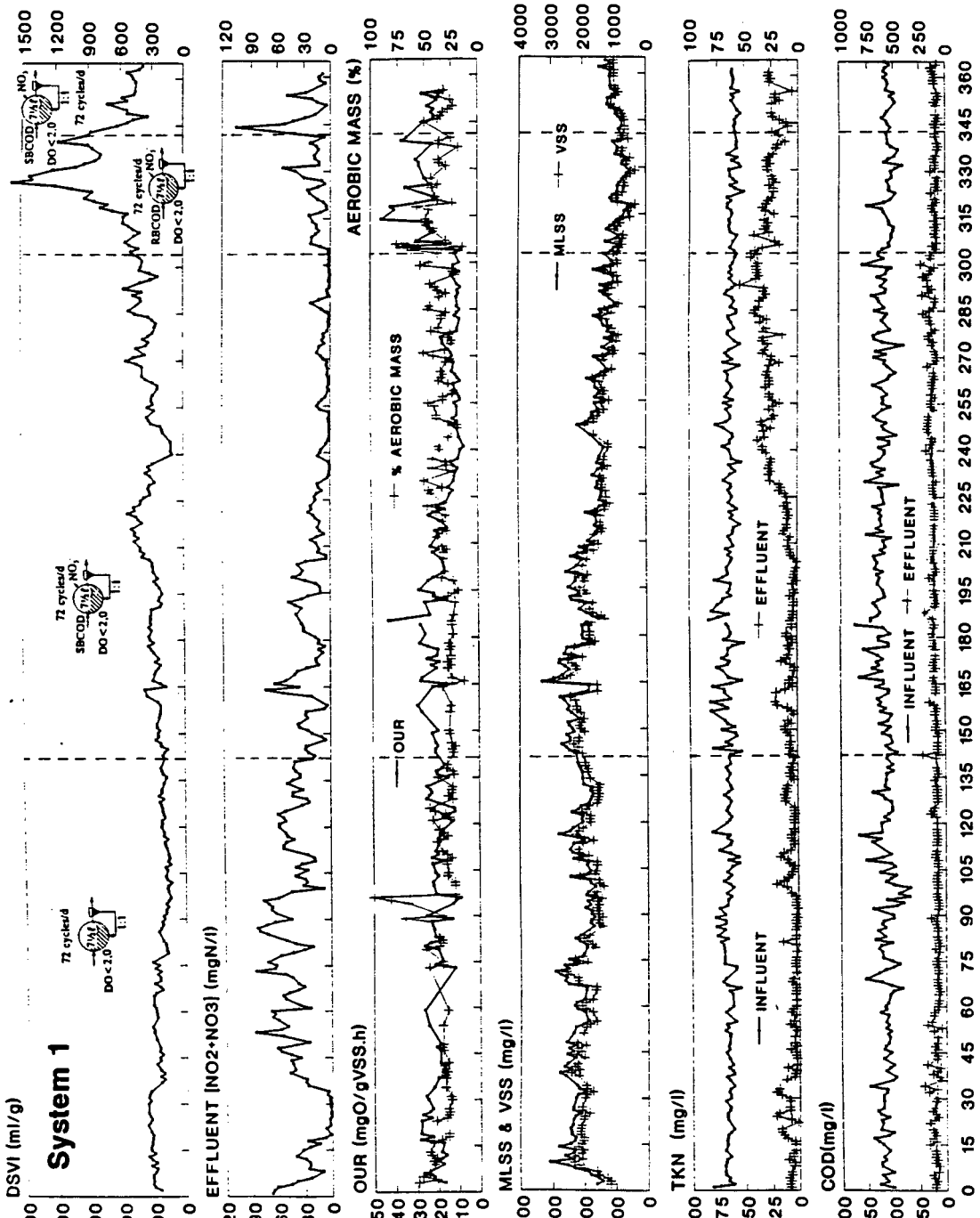


Fig A.2: Daily measured data for System 1.

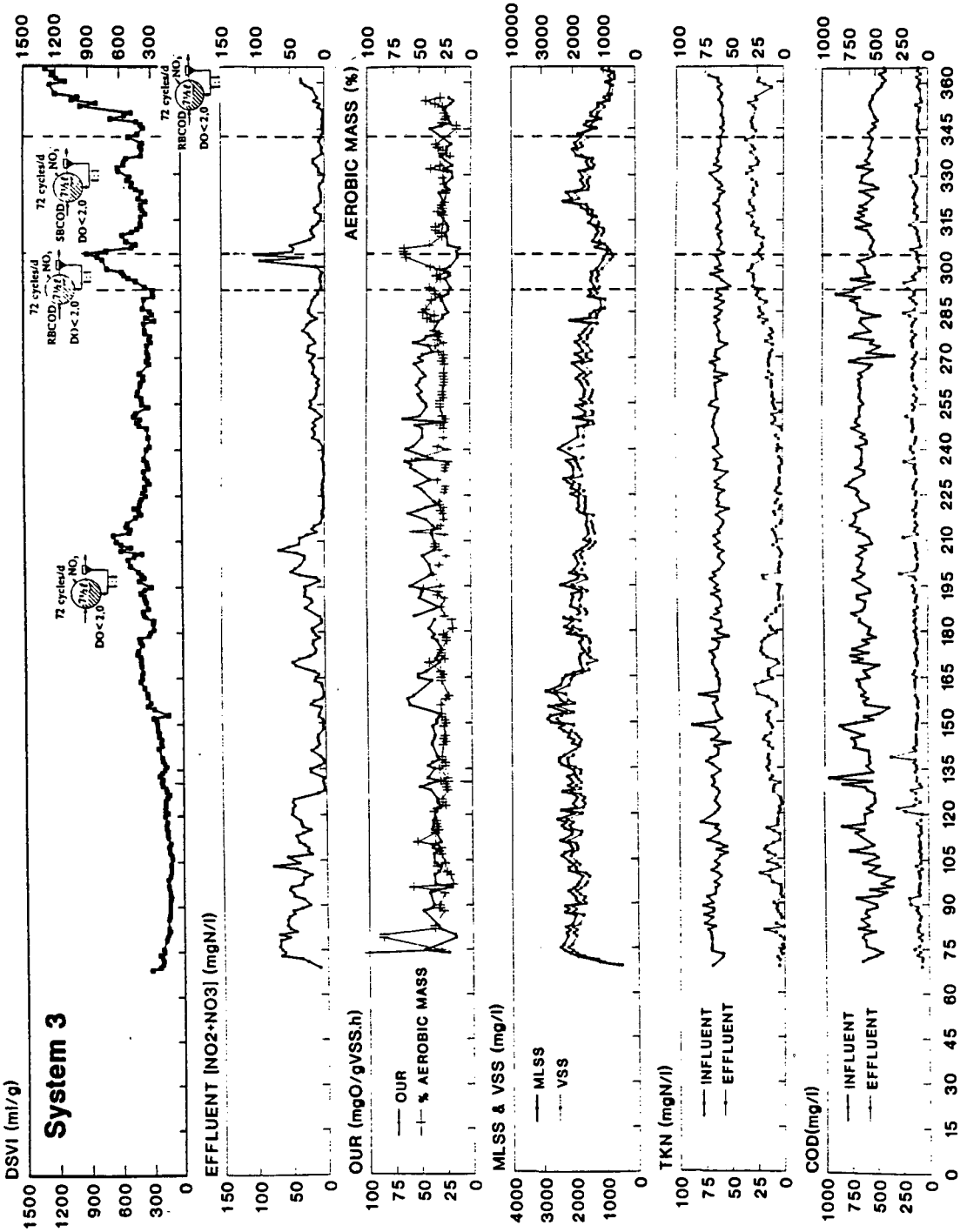


Fig A.4: Daily measured data for System 3.

Table A.2: Mass balances of COD and Nitrogen for System 1.

COD & NITROGEN Balance System 1

Q =	10	l/d
Vp =	7.5	l
Rs =	15	d
w =	500	ml
fn =	0.1	mgN/mgVSS
fcv =	1.48	mgCOD/mgVSS

MSti: influent COD
MSte: effluent COD
Xv: volatile solids
MZw: sludge wasted
MOc: carbonac.O2 demand
MNti: influent nitrogen
MNte: effluent nitrogen
MNw: waste sludge nitrogen
MNoxi: nitrate/nitrite added

MNoxd: nitrate denitrified
MNoxn: nitrate generated by nitrification
%Aer: Percentage aerobic
MOt: total O2 consumed
MOn: nitrification O2 requirement
MOh: heterotrophic O2 requirement
MOd: denitrification O2 equivalent
MNoxe: effluent nitrate

Steady State Period	Day-Day	MSti mg/d	MSte mg/d	Xv mg/l	MZw mg/d	MOc mgO/d	COD Mass Balance	Steady State Period
1	1 31	5968	1086	1759	1302	2839	88	1
2	32 84	5675	914	2034	1505	2356	84	2
3	85 141	5586	833	1747	1293	1188	59	3
4	142 216	6144	989	1981	1466	1862	70	4
5	217 259	5986	1160	1393	1031	1191	57	5
6	260 303	5790	1283	1131	837	1127	56	6
7	304 341	5348	905	701	519	1402	53	7
8	342 365	5489	905	912	675	735	42	8

Steady State Period	Day-Day	MNti	MNte	MNw	MNoxi	MNoxe	MNoxd	MNoxn	Nitrogen Mass Balance	Steady State Period
1	1 31	675	98	88	211	229	472	489	100	1
2	32 84	660	53	102	485	452	538	505	100	2
3	85 141	655	60	87	269	464	312	507	100	3
4	142 216	647	70	99	247	246	479	478	100	4
5	217 259	630	223	70	261	92	507	338	100	5
6	260 303	618	323	57	258	50	447	239	100	6
7	304 341	604	261	35	254	164	398	308	100	7
8	342 365	590	204	46	266	295	312	340	100	8

Steady State Period	Day-Day	OUR mgO/l.h	%Aer	MOt mgO/d	MOn mgO/d	MOh mgO/d	MOd mgO/d	Steady State Period
1	1 31	43.7	47	3725	2235	1490	1349	1
2	32 84	40.7	43	3127	2309	818	1538	2
3	85 141	32.7	44	2614	2319	295	893	3
4	142 216	44.5	33	2675	2184	491	1371	4
5	217 259	17.1	42	1288	1546	-258	1449	5
6	260 303	12.5	42	941	1092	-151	1278	6
7	304 341	19.4	48	1669	1406	263	1140	7
8	342 365	19.1	41	1399	1556	-157	892	8

Table A.4: Mass balances of COD and Nitrogen for System 3.

COD & NITROGEN Balance System 3

Q =	10	l/d
Vp =	7.5	l
Rs =	15	d
w =	.500	ml
fn =	0.1	mgN/mgVSS
fcv =	1.48	mgCOD/mgVSS

MSti: influent COD
MSte: effluent COD
Xv: volatile solids
MZw: sludge wasted
MOc: carbonac.O2 demand
MNti: influent nitrogen
MNte: effluent nitrogen
MNw: waste sludge nitrogen
MNoxi: nitrate/nitrite added
MNoxd: nitrate denitrified
MNoxn: nitrate generated by nitrification
%Aer: Percentage aerobic
MOt: total O2 consumed
MOn: nitrification O2 requirement
MOh: heterotrophic O2 requirement
MOd: denitrification O2 equivalent
MNoxe: effluent nitrate

Steady State Period	Day-Day	MSti mg/d	MSte mg/d	Xv mg/l	MZw mg/d	MOc mgO/d	COD Mass Balance	Steady State Period
1	1 66	5621	988	1838	1360	1409	67	1
2	67 103	6287	1143	1907	1411	1954	72	2
3	104 149	6230	1232	1681	1244	1860	70	3
4	150 169	6463	1148	1670	1236	2752	79	4
5	170 222	6404	1248	1448	1072	2141	70	5
6	223 259	6079	1264	1413	1045	2173	74	6
7	260 297	6061	1139	1051	778	1114	50	7
8	298 342	5746	1034	1401	1037	1420	61	8
9	343 365	4883	833	1012	749	1291	59	9

Steady State Period	Day-Day	MNti	MNte	MNw	MNoxi	MNoxe	MNoxd	MNoxn	Nitrogen Mass Balance	Steady State Period
1	1 66	665	73	92	224	421	303	500	100	1
2	67 103	654	145	95	237	125	525	413	100	2
3	104 149	606	69	84	250	273	430	453	100	3
4	150 169	609	40	83	247	40	692	486	100	4
5	170 222	652	72	72	239	145	601	507	100	5
6	223 259	622	151	71	258	173	486	401	100	6
7	260 297	600	266	53	248	276	254	282	100	7
8	298 342	621	279	70	242	129	386	272	100	8
9	343 365	605	262	51	246	105	433	293	100	9

Steady State Period	Day-Day	OUR mgO/l.h	%Aer	MOt mgO/d	MOn mgO/d	MOh mgO/d	MOd mgO/d	Steady State Period
1	1 129	34.9	45	2826	2283	543	867	1
2	130 178	39.1	33	2340	1888	453	1502	2
3	179 212	42.4	35	2698	2069	629	1231	3
4	213 244	47.1	35	2991	2219	772	1980	4
5	245 259	46.8	33	2740	2317	423	1718	5
6	260 291	38.6	38	2615	1832	783	1391	6
7	292 304	19.8	47	1674	1287	387	727	7
8	305 341	22.9	38	1562	1245	317	1103	8
9	342 365	24.0	32	1391	1337	54	1238	9

Table A.6: Changes in operating conditions and their effect on system response for System 1.

SYSTEM 1 :		CHANGES IN OPERATING CONDITIONS
Period (Days)	Operating conditions	Reasons for changes in operating conditions: Comments on system response
1 - 32	Intermittent aeration (0,2 < DO < 2,0) Average substrate Continuous NO_3^- addition	To develop a sludge with high DSVI containing *M.parvicella*: DSVI remained between 300 and 350 ml/g. Dominant filaments were type 0092, 0914 and *H.hydrossis*. *M.parvicella* secondary at start then reduced to tertiary.
33 - 141	Intermittent aeration (0,2 < DO < 2,0) Average substrate without fats and oils Continuous NO_3^- addition	To determine effect of lack of fats and oils on *M.parvicella*: DSVI decreased from ≈ 350 to ≈ 200 ml/g; *H.hydrossis* was dominant, type 0092 the secondary filament.
142 - 303	Intermittent aeration (0,2 < DO < 2,0) SBCOD-rich substrate Continuous NO_3^- addition	To determine if filaments proliferate with SBCOD and as a complement to System 3 (Days 292-303): DSVI increased from ≈ 200 to ≈ 450 ml/g and *H.hydrossis* and type 1851 were dominant. Decrease in DSVI on Day 238 due to solids loss resulting in high percent aerobic fraction (> 40%). Note the low COD balance as the DSVI increased (see Fig. 3.1), most likely due to solids loss in effluent.
304 - 341	Intermittent aeration (0,2 < DO < 2,0) RBCOD-rich substrate Continuous NO_3^- addition	To determine if filaments proliferate with RBCOD and as a complement to System 3 (Days 304-341): DSVI increased from ≈ 450 to ≈ 1200 ml/g and *H.hydrossis* was dominant. Note the very low COD mass balance (53%) due to high DSVI values and loss of solids in effluent.
342 - 365	Intermittent aeration (0,2 < DO < 2,0) SBCOD-rich substrate Continuous NO_3^- addition	To determine if filaments proliferate with SBCOD and as a complement to System 3 (Days 342-365): DSVI decreased from ≈ 1200 to ≈ 400 ml/g; the dominant filament was *H.hydrossis*.

Summary:

- *M.parvicella* did not proliferate with fats and oils in the substrate
- With average substrate composition, *H.hydrossis* and type 1851 were dominant
- With RBCOD-substrate, DSVI increased and *H.hydrossis* and type 1851 were dominant
- With SBCOD-substrate, DSVI decreased and filaments *H.hydrossis* and type 1851 were dominant
- In changing between substrate types (RBCOD to SBCOD), filament type 1701 increased in abundance

Table A.8: Changes in operating conditions and their effect on system response for System 3.

SYSTEM 3 :		CHANGES IN OPERATING CONDITIONS
Period (Days)	Operating conditions	Reasons for changes in operating conditions: Comments on system response
69 - 129	Intermittent aeration (0,2 < DO < 2,0) average substrate - without Fats & Oils Continuous NO_3^- addition	To develop a sludge with high DSVI containing *M.parvicella*: DSVI remained 150-200 ml/g; dominant filaments were *H.hydrossis* and type 1851. *M.parvicella* was not identified.
130 - 291	Intermittent aeration (0,2 < DO < 2,0) Average substrate - with Fats & Oils Continuous NO_3^- addition	To determine if addition of Fats & Oils induces *M.parvicella* growth: DSVI fluctuated between 200 and 650 ml/g and *H.hydrossis* and type 1851 were dominant filaments. Increases and decreases in DSVI are related to increases and decreases in effluent $NO_{3,3}^-$ concentrations (see Fig 3.5) in particular between Days 212 and 365. Note that the COD mass balance decreased when the DSVI was decreasing (Days 213-244, 79%) and was less than when DSVI was increasing (Days 179-212, 70%) (see Table A.4).
292 - 303	Intermittent aeration (0,2 < DO < 2,0) RBCOD-rich substrate Continuous NO_3^- addition	To determine if filaments proliferate with RBCOD and as a complement to System 1 (Days 142-303): DSVI increased from ≈ 280 to ≈ 900 ml/g; type 1851 was dominant and *H.hydrossis* secondary. Note the very low COD balance (50%).
304 - 341	Intermittent aeration (0,2 < DO < 2,0) SBCOD-rich substrate Continuous NO_3^- addition	To determine if filaments proliferate with SBCOD and as a complement to System 1 (Days 304-341): DSVI decreased from ≈ 900 to ≈ 380 ml/g; *H.hydrossis* was the dominant filament.
342 - 365	Intermittent aeration (0,2 < DO < 2,0) RBCOD-rich substrate Continuous NO_3^- addition	To determine if filaments proliferate with RBCOD and as a complement to System 1 (Days 342-365): DSVI increased from ≈ 380 to ≈ 1300 ml/g; *H.hydrossis* and type 1851 were dominant filaments.

Summary:

- *M.parvicella* did not proliferate with fats and oils
- With average substrate composition, *H.hydrossis* and type 1851 were dominant
- With RBCOD-rich substrate, DSVI increased and *H.hydrossis* and type 1851 were dominant
- With SBCOD-rich substrate, DSVI decreased

Table A.10: Volumes of suspensions for inclusion in substrate feed types A, B, C, D, and E.

VOLUMES OF SUSPENSIONS FOR DIFFERENT SUBSTRATE FEED TYPES					
SUBSTRATE SUSPENSIONS	Substrate feed types and volumes (ml)				
	A	B	C	D	E
RBCOD	20	20	-	140	20
Complex carbohydrates	170	300	360	-	300
Fats and oils	140	-	-	-	-
Organic nitrogen	140	140	140	140	140
Vitamins	30	30	30	30	30
Macro-inorganic nutrients 1	170	170	170	170	-
Macro-inorganic nutrients 2	-	-	-	-	170
Micro-inorganic nutrients 1	30	30	30	30	30
Micro-inorganic nutrients 2	50	50	50	50	50

Table A.11: Periods for different substrate types fed to Systems 1 to 4.

PERIODS DURING WHICH DIFFERENT SUBSTRATE TYPES FED TO SYSTEMS 1-4			
SYSTEM 1	SYSTEM 2	SYSTEM 3	SYSTEM 4
1 - 31 (A)	1 - 66 (A)	1 - 129 (B)	1 - 141 (B)
32 - 141 (B)	67 - 297 (B)	130 - 291 (A)	142 - 365 (D)
142 - 303 (C)	298 - 342 (E)	292 - 303 (D)	
304 - 341 (D)	343 - 380 (B)	304 - 341 (C)	
342 - 365 (C)		342 - 365 (D)	

aerobic period. For this purpose, time periods of 20–60 days were chosen during which the reactor configuration and aeration pattern did not change. The average percentage aerobic period was calculated over the chosen time period but the corresponding average DSVI was calculated over a much shorter time at the end of this period when the DSVI had reached a steady–state value. As can be seen in Fig A.3, for System 2, the DSVI changes quite considerably over the chosen time periods and for this reason the average DSVI value is calculated only over a time period at the end of the steady state period over which the value of the average percentage aerobic period is calculated. Figure A 6 illustrates the relationship between DSVI and percentage aerobic period for System 2. Table A.13 indicates the time periods over which the percentage aerobic periods and DSVIs were determined. The investigation was conducted on System 2 because of its long steady–state periods; Systems 1, 3 and 4 experienced regular changes in configuration, aeration pattern and/or substrate composition which excluded a similar analysis being conducted on them.

Effect of system configuration, aeration pattern, nitrate concentration and substrate composition on the proliferation of filamentous organism types

Filament identifications with time for Systems 1 to 4 are given in Tables A.14 to A.17 respectively. The effects of system configuration, aeration pattern, nitrate concentration and substrate composition on the type of filamentous organisms which develop and extent of their proliferation in systems fed defined substrate are summarized in Table A.18. The effects of these changes on filamentous organisms not identified at the Dominant or Secondary abundance levels is difficult to ascertain and are not reported on. It should be noted that the filaments which proliferate on a particular defined artificial substrate composition under specific conditions and may not necessarily proliferate in systems fed municipal sewage with similar configurations and aeration patterns.

A physical effect of the "Fats & Oils" suspension of the defined substrate on the settleability of sludge during the DSVI test

As a result of the finding in Systems 1, 2 and 3 that the substrate suspension Fats & Oils does not affect the proliferation of *Microthrix parvicella*, this suspension was excluded from subsequent substrate compositions. However, although Fats & Oils did not appear to affect the type of filament which proliferated in the systems, small changes in settleability of the sludge (DSVI) were noticed after removal of the Fats & Oils suspension from the substrate (System 1, Day 32) and after addition of the

Table A.14: Filament identifications with time for System 1.

Day No. System 1	Filament abundance	Dominant	Secondary	Tertiary	Remarks
9		0092	*M.parvicella*	0041, 0675, Flexibacter, *N.limicola II*	
25		0092	0914	1851, *M.parvicella*, 0041	
58		*H.hydrossis*	0092	0803, 0041, *M.parvicella*	
94		*H.hydrossis*	0092	0675, 0041, Flexibacter	•strings of beads
107	common-very common	*H.hydrossis*	0092	1851, 0675, 0041	
128	common-very common	*H.hydrossis*	0092	0041, 1851	
144	very common	*H.hydrossis*	0092	0041, 1851, 0914	•strings of beads
170	very common	*H.hydrossis*	1851	0914, 0041, 0092	•strings of beads
192	common-very common	1851	*H.hydrossis*	0092, *Thiothrix I*, 0914, 0041	•strings of beads
213	common-very common	1851, *H.hydrossis*	-	0092, 0041, *Beggiatoa*, 0914, *N.limicola II*	
263	common	*H.hydrossis*	1851	021N, 1701, 0041, Flexibacter, *Beggiatoa*	
304	common-very common	*H.hydrossis*	1851	1701, 0041	
334	very common-abundant	*H.hydrossis*	1701	021N, 0092, 1851	
365	abundant	*H.hydrossis*	-	Flexibacter, 0041, 1851, 1701	•some *algae* sp. present

Table A.16: Filament identifications with time for System 3

Day No. System 3	Filament abundance	Dominant	Secondary	Tertiary	Remarks
74		*H.hydrossis*, 0092	-	0675, 0041, Flexibacter, 1851, *N.limicola I*	
107	common	*H.hydrossis*	1851	0092, 0041, *M.parvicella*	
128	common	*H.hydrossis*	1851	0041	•Rotifers present
144	very common	*H.hydrossis*	1851	0041	
170	very common-abundant	*H.hydrossis*	1851	*Beggiatoa*, 1863, 0041, 0092, *N.limicola II*	
192	very common-abundant	*H.hydrossis, 1851*	-	1863, 0041, strings of beads, 0092	
213	common-very common	*H.hydrossis*	1851	0041, 0092	
263	very common-abundant	1851, *H.hydrossis*	-	0092, 0041, 1863, *N.limicola II, Beggiatoa*	•unidentified
304	very common-abundant	*1851*	*H.hydrossis*	0092, 0041, *N.limicola III*	filaments •chains of large
334	abundant	*H.hydrossis*	1851	0041, *1701, 0092*	ovoid cells
363	abundant	*H.hydrossis*	1851	0041, *Thiothrix* sp., 0803	

Table A.18: Effect of system configuration, aeration pattern, and substrate composition on the type of filamentous organisms which develop and their extent of proliferation in Systems 1–4 fed defined artificial substrate.

THE EFFECT OF VARIOUS CONDITIONS ON FILAMENTOUS ORGANISMS DEVELOPED ON DEFINED ARTIFICIAL SUBSTRATE			
ORGANISM TYPE	**Conditions which promote or sustain proliferation**	**Conditions which reduce proliferation**	**Conditions which have little or no effect on proliferation**
Haliscomenobacter hydrossis	• Intermittent aeration + RBCOD-rich substrate or SBCOD-rich substrate	• Fully aerobic • Low [NO] (<5,0 mgN/ℓ) • Aerobic selectors + RBCOD-rich substrate • Anoxic selectors + RBCOD-rich substrate	• FATS & OILS present or absent
1851	• Intermittent aeration • RBCOD-rich substrate • SBCOD-rich substrate	• Fully aerobic • FATS & OILS absent • Aerobic selectors + RBCOD-rich substrate	• FATS & OILS present or absent
0092		• SBCOD-rich substrate	• FATS & OILS absent
1701	• RBCOD-rich substrate • Aerobic selectors + RBCOD-rich substrate • Anoxic selectors + RBCOD-rich substrate • Low [NO] (<5,0 mgN/ℓ)	• SBCOD-rich substrate	• Fully aerobic
0041			• Fully aerobic
Microthrix parvicella		• Intermittent aeration	• Fully aerobic
0914		• Intermittent aeration	
0803	• Anoxic selectors + RBCOD-rich substrate		

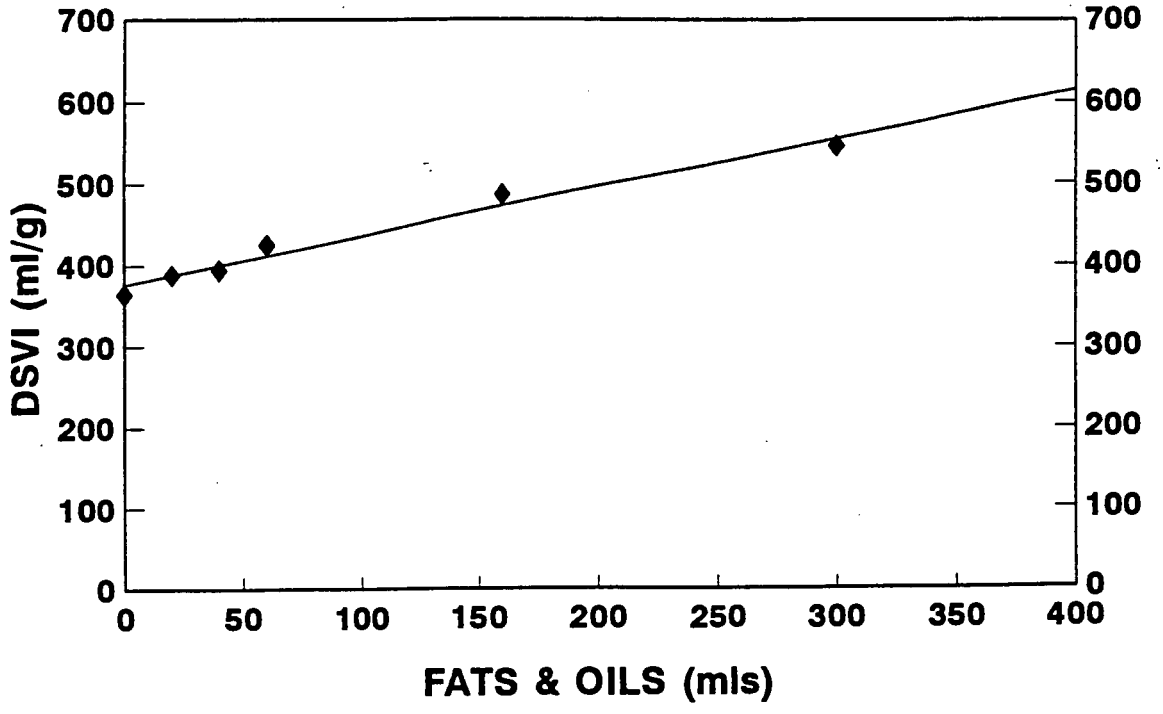
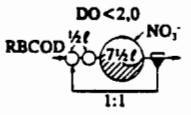


Fig A.7: The physical effect of different volumes of Fats & Oils (mℓ) on sludge settleability (DSVI in mℓ/g).

Table A.19: Operating conditions for Defined Substrate Batch Test 1 (DSBT1).

BATCH TEST DSBT1 OPERATING CONDITIONS		
Objective: To examine extent of induction of selector effect in system with 2 aerobic selectors fed RBCOD.		
PARENT SYSTEM CONDITIONS		
System 4; Day 194 **System configuration:IAND & 2 aerobic selectors** **Substrate type: RBCOD**		
Sludge age	**(d)**	**15**
BATCH TEST CONDITIONS		
Total volume	**(ℓ)**	**3,0**
MLSS	**(mg/ℓ)**	**443**
VSS	**(mg/ℓ)**	**333**
F/M	**(mgCOD/mgVSS**	**0,85**
DO	**(mgO/ℓ)**	**2 - 4**
Temperature	**(°C)**	**20**
SUBSTRATE TO BATCH TEST		
RBCOD	**(mgCOD/ℓ-final batch vol)**	**284**

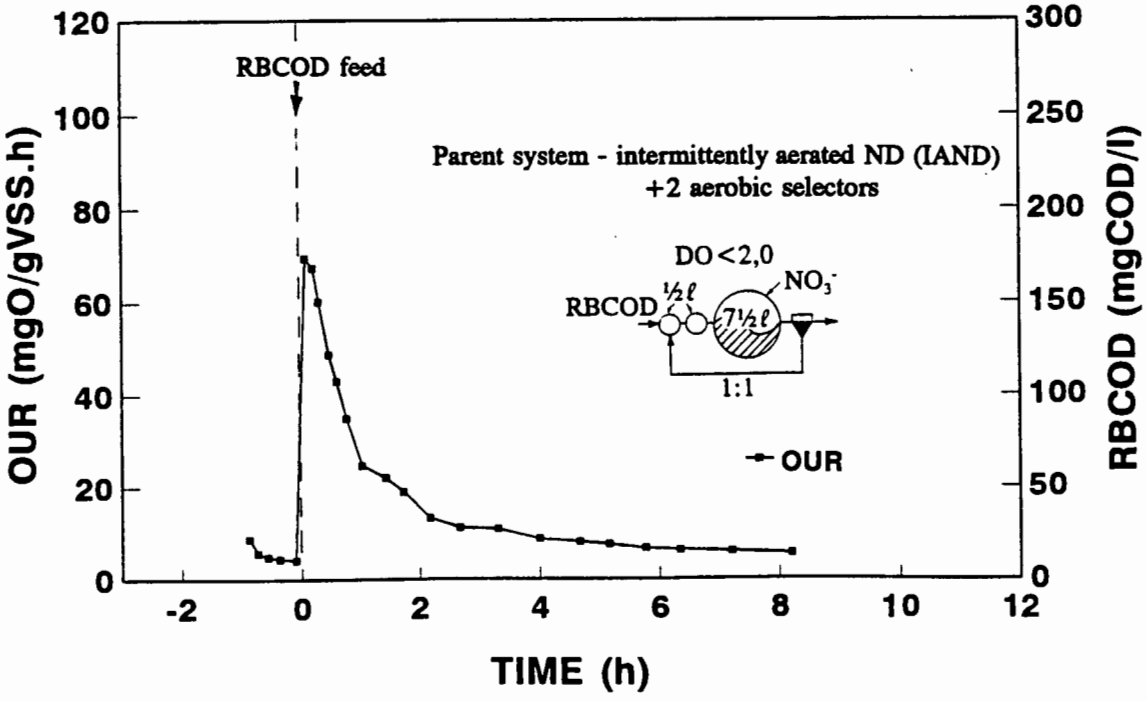
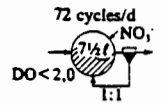


Fig A.8: Oxygen utilization rate (OUR in mgO/gVSS.h) with time (h) for Defined Substrate Batch Test 1 (DSBT1) conducted on Day 194 on sludge from System 4.

Table A.21: Operating conditions for Defined Substrate Batch Test 3 (DSBT3).

BATCH TEST DSBT3 OPERATING CONDITIONS		
Objective: To examine extent of induction of selector effect in system fed average substrate.		
PARENT SYSTEM CONDITIONS		
System 3; Day 199 System configuration: IAND Substrate type: Average COD		
Sludge age	(d)	15
BATCH TEST CONDITIONS		
Total volume	(ℓ)	3,0
MLSS	(mg/ℓ)	663
VSS	(mg/ℓ)	580
F/M	(mgCOD/mgVSS	0,70
DO	(mgO/ℓ)	2 - 4
Temperature	(°C)	20
SUBSTRATE TO BATCH TEST		
RBCOD	(mgCOD/ℓ-final batch vol)	404

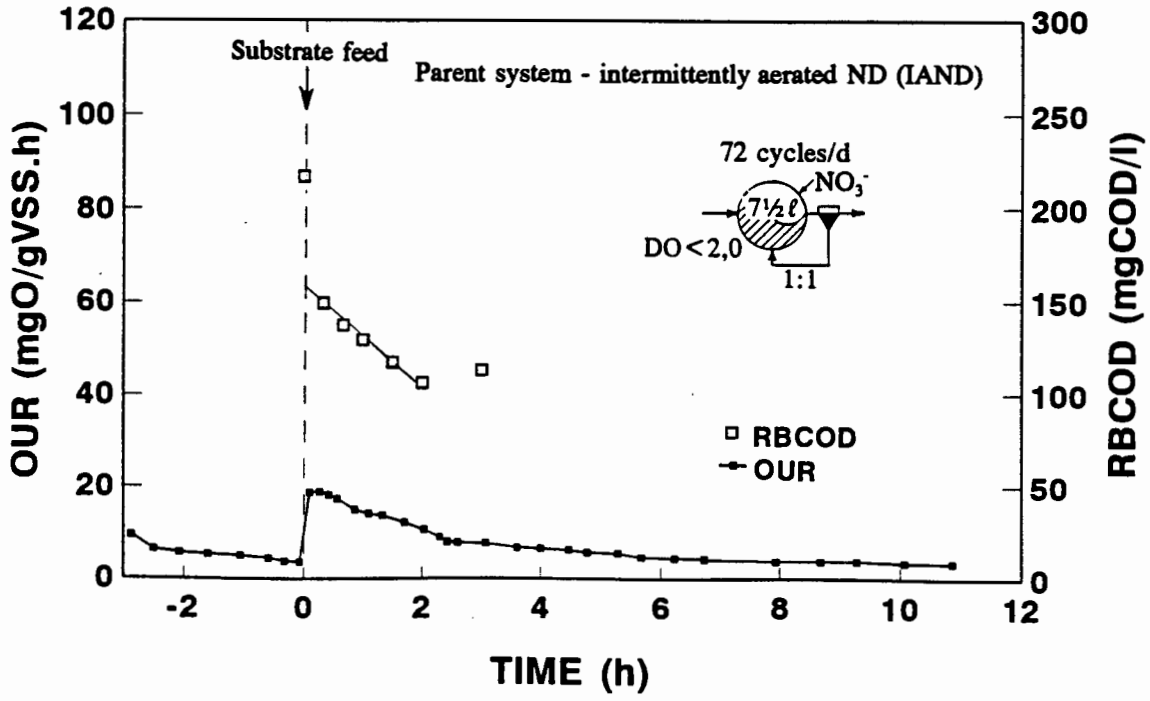
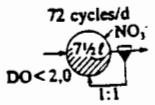


Fig A.10: Oxygen utilization rate (OUR in mgO/gVSS.h) with time (h) for Defined Substrate Batch Test 3 (DSBT3) conducted on Day 199 on sludge from System 3.

Table A.23: Operating conditions for Defined Substrate Batch Test 5 (DSBT5).

BATCH TEST DSBT5 OPERATING CONDITIONS		
Objective: To examine extent of induction of selector effect in system fed SBCOD.		
PARENT SYSTEM CONDITIONS		
System 2; Day 205 System configuration: IAND Substrate type: Average COD		
Sludge age	(d)	15
BATCH TEST CONDITIONS		
Total volume	(ℓ)	3,0
MLSS	(mg/ℓ)	749
VSS	(mg/ℓ)	676
F/M	(mgCOD/mgVSS	0,48
DO	(mgO/ℓ)	2 - 4
Temperature	(°C)	20
SUBSTRATE TO BATCH TEST		
RBCOD	(mgCOD/ℓ-final batch vol)	324

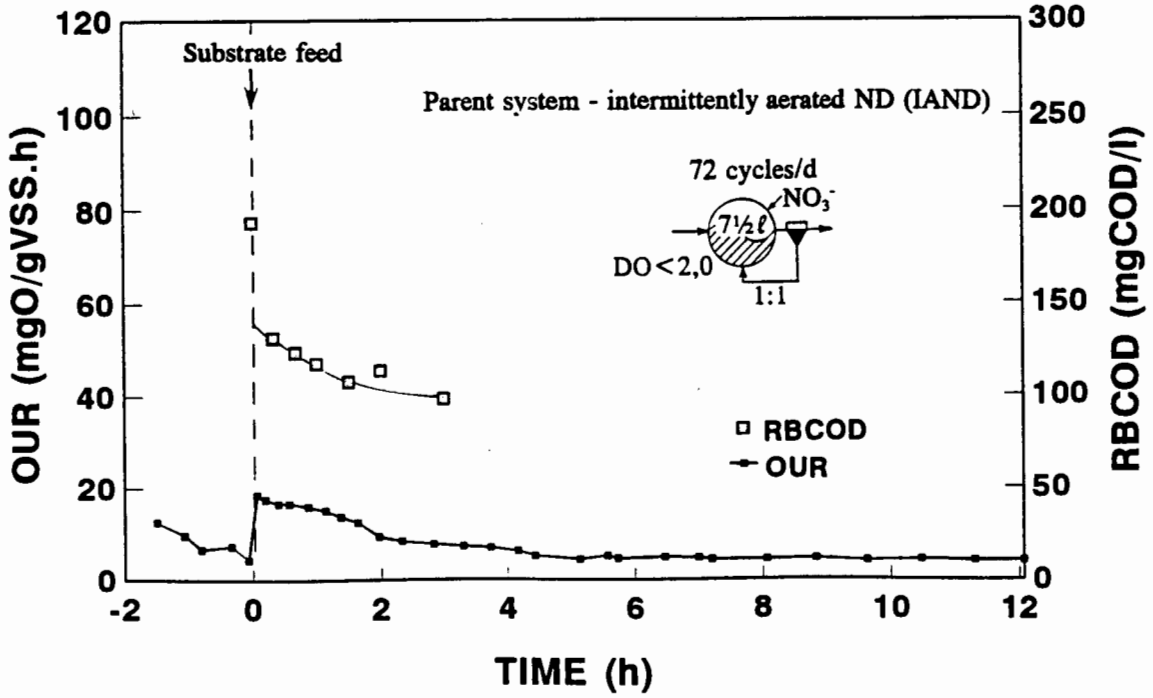
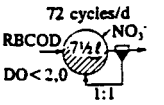


Fig A.12: Oxygen utilization rate (OUR in mgO/gVSS.h) with time (h) for Defined Substrate Batch Test 5 (DSBT5) conducted on Day 205 on sludge from System 2.

Table A.25: Operating conditions for Defined Substrate Batch Test 7 (DSBT7).

BATCH TEST DSBT7 OPERATING CONDITIONS		
Objective: To examine the role of RBCOD in inducing a selector effect.		
PARENT SYSTEM CONDITIONS		
System 4; Day 222 System configuration: IAND Substrate type: RBCOD		
Sludge age	(d)	15
BATCH TEST CONDITIONS		
Total volume	(ℓ)	3,0
MLSS	(mg/ℓ)	363
VSS	(mg/ℓ)	322
F/M	(mgCOD/mgVSS	0,66
DO	(mgO/ℓ)	2 - 4
Temperature	(°C)	20
SUBSTRATE TO BATCH TEST		
RBCOD	(mgCOD/ℓ-final batch vol)	211

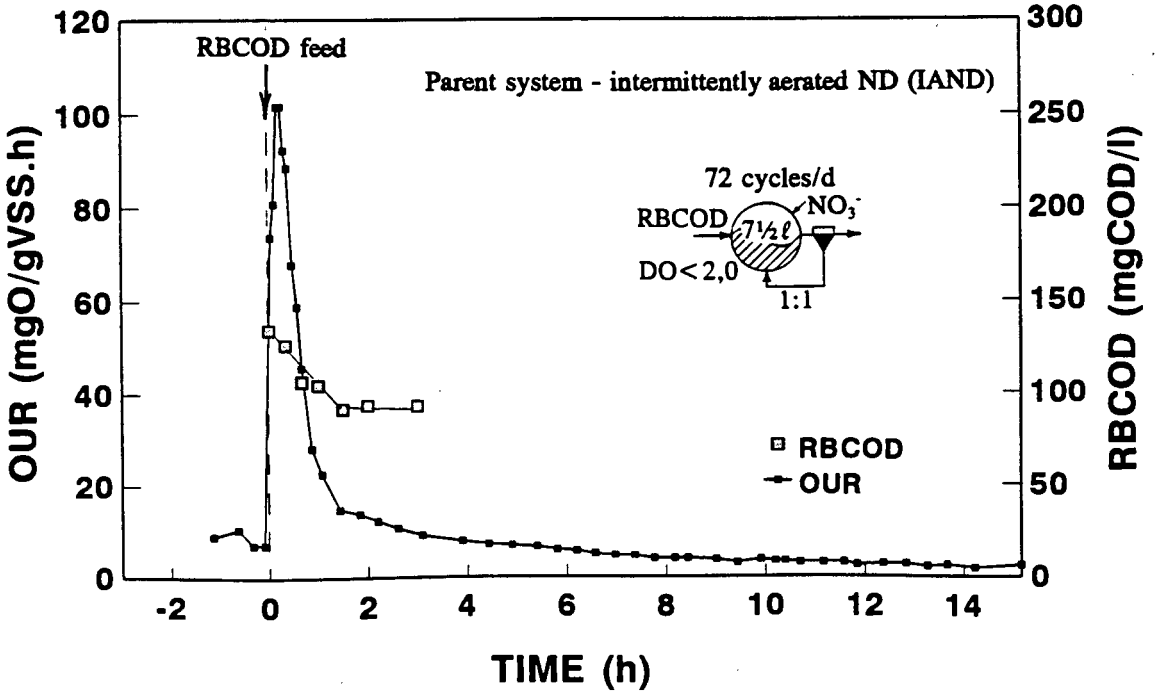
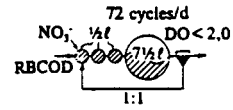


Fig A.14: Oxygen utilization rate (OUR in mgO/gVSS.h) with time (h) for Defined Substrate Batch Test 7 (DSBT7) conducted on Day 222 on sludge from System 4.

Table A.27: Operating conditions for Defined Substrate Batch Test 9 (DSBT9).

BATCH TEST DSBT9 OPERATING CONDITIONS		
Objective: To examine the extent of induction of selector effect in system with 3 anoxic selectors.		
PARENT SYSTEM CONDITIONS		
System 4; Day 326 System configuration:IAND & 3 anoxic selectors Substrate type: RBCOD		
Sludge age	(d)	15
BATCH TEST CONDITIONS		
Total volume	(ℓ)	3,0
MLSS	(mg/ℓ)	526
VSS	(mg/ℓ)	392
F/M	(mgCOD/mgVSS	0,88
DO	(mgO/ℓ)	2 - 4
Temperature	(°C)	20
SUBSTRATE TO BATCH TEST		
RBCOD	(mgCOD/ℓ-final batch vol)	346

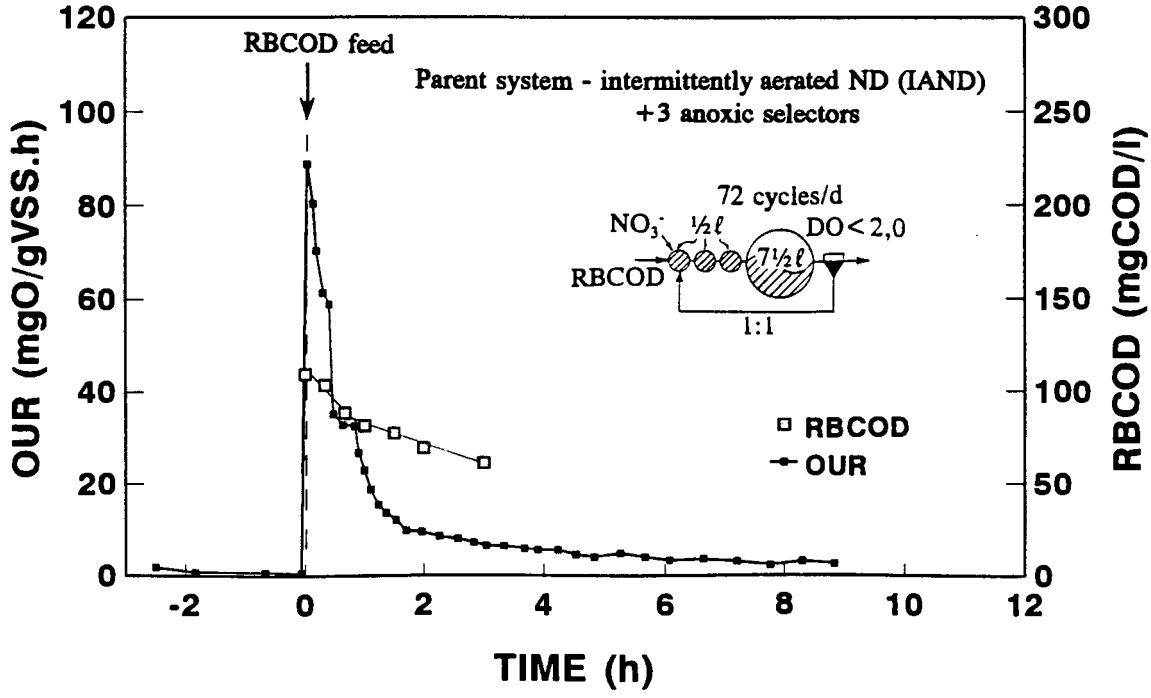
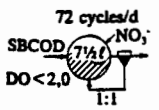


Fig A.16: Oxygen utilization rate (OUR in mgO/gVSS.h) with time (h) for Defined Substrate Batch Test 9 (DSBT9) conducted on Day 326 on sludge from System 4.

Table A.29: Operating conditions for Defined Substrate Batch Test 11 (DSBT11).

BATCH TEST DSBT11 OPERATING CONDITIONS		
Objective: To examine the extent of induction of selector effect in IAND system fed SBCOD.		
PARENT SYSTEM CONDITIONS		
System 3; Day 334 System configuration: IAND Substrate type: SBCOD		
Sludge age	(d)	15
BATCH TEST CONDITIONS		
Total volume	(ℓ)	3,0
MLSS	(mg/ℓ)	562
VSS	(mg/ℓ)	481
F/M	(mgCOD/mgVSS	0,72
DO	(mgO/ℓ)	2 - 4
Temperature	(°C)	20
SUBSTRATE TO BATCH TEST		
RBCOD	(mgCOD/ℓ-final batch vol)	346

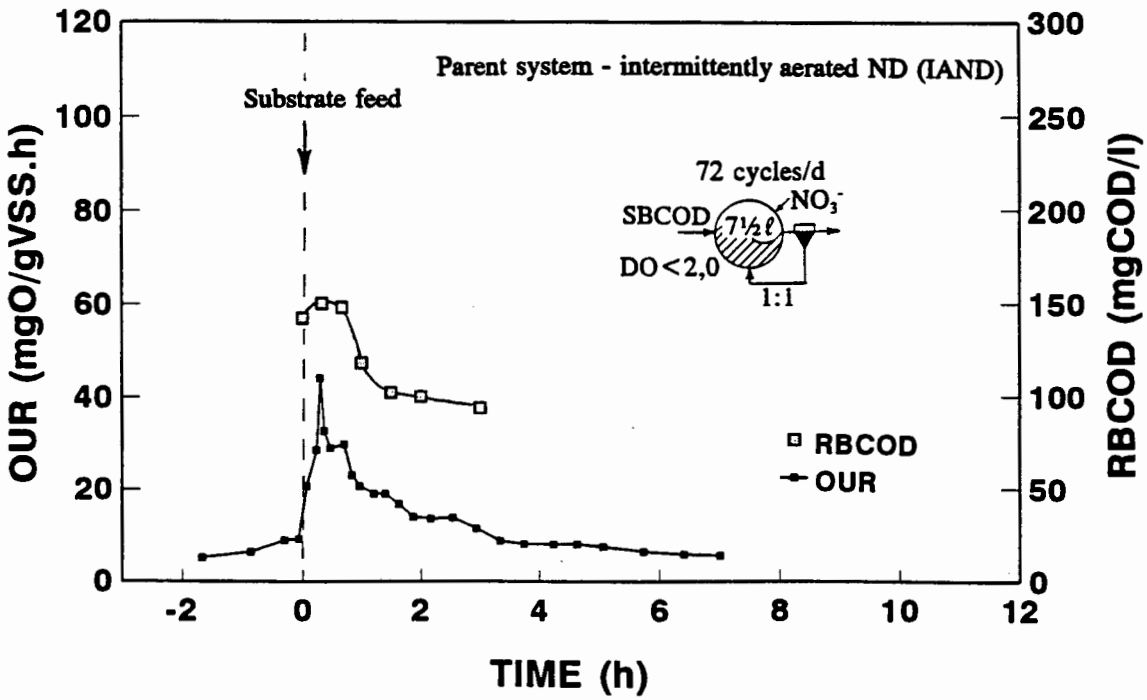


Fig A.18: Oxygen utilization rate (OUR in mgO/gVSS.h) with time (h) for Defined Substrate Batch Test 11 (DSBT11) conducted on Day 334 on sludge from System 3.

Induction of the selector effect by anoxic selector reactor configuration

Three aerobic batch tests were conducted on System 4 during the period it incorporated 2 or 3 anoxic selector reactors: DSBT7 (Day 286 – selector effect measured); DSBT8 (Day 324 – selector effect measured); and DSBT9 (Day 324 – selector effect measured). As for the conclusions regarding induction of the selector effect by aerobic selectors, it is unclear as to whether the selector effect is a consequence of the anoxic selector reactor configuration, or the RBCOD–rich substrate fed to the system. However it is interesting to note that although the RBCOD–rich substrate was utilized under *anoxic* conditions in the 2 and 3 anoxic selector–reactor configurations, high substrate utilization rates were measured under *aerobic* batch test conditions.

To determine the efficacy of the anoxic selector reactors in removing RBCOD, three COD profiles were conducted when System 4 incorporated 2 anoxic selectors and eight COD profiles when System 4 incorporated 3 anoxic selectors. The results of the profiles for System 4, with 2 and 3 anoxic selectors are given in Table A.31 and Table A.32 respectively and indicate that 90% and 92% of the COD was utilized in the first selector reactor of the 2 and 3 anoxic selector reactor configurations respectively.

Selector effect removed with time under non–inductive conditions

An apparently anomalous result was measured for DSBT11 (System 3, Day 334) in which System 3 at the time of the batch test was being fed *particulate* substrate and a partial selector effect was measured. This can be attributed to the system being fed RBCOD–rich substrate for a period of a sludge age, 2 sludge ages previously. This conforms with the findings of Still *et al.* (1986) in which a period of 3 sludge ages was required for complete removal of the selector effect.

A.6 DETERMINATION OF THE INFLUENT RBCOD FRACTION OF THE DEFINED SUBSTRATE

Introduction

After operation of IAND Systems 1–4 for more than 3 sludge ages it was recognized that filament proliferation with artificial substrate (DSVI $\approx$ 500 mℓ/g) was far greater than filament proliferation in configurations with the same conditions but fed municipal sewage (DSVI $\approx$ 250 mℓ/g).

It was concluded that certain characteristics of the artificial substrate were

responsible for the greater proliferation of the filamentous organisms than with municipal sewage. According to the proposal of Chudoba (1985) the concentration of RBCOD in a system is of particular importance in the proliferation of filamentous organisms and attention was directed at determining the proportions of RBCOD and SBCOD in the artificial substrate to ascertain if a significant difference could be determined between the RBCOD fractions of the defined substrate and municipal sewage.

Background

The COD of municipal wastewaters has been categorized into two main fractions; biodegradable and unbiodegradable. Each fraction has 2 subfractions; for the biodegradable COD, these are soluble readily biodegradable and particulate slowly biodegradable, and for the unbiodegradable COD, soluble unbiodegradable and particulate unbiodegradable. The subdivision of biodegradable COD into soluble readily biodegradable COD (RBCOD) and particulate slowly biodegradable COD (SBCOD) is made on the basis of biological response to the aerobic batch test, as described in Section A.1.

Determination of the RBCOD fraction of artificial substrate as influent to the activated sludge process is essential for the proper operation of activated sludge systems for two reasons; (i) the concentration of RBCOD in the reactor has been hypothesized to play a role in filamentous organism proliferation (Chudoba, 1985) and therefore by implication so also the fraction of RBCOD in the influent, and (ii) research at the University of Cape Town has indicated that knowledge of the proportions of RBCOD and SBCOD in a wastewater or substrate is essential for accurate modelling of the behaviour of the activated sludge process to which the wastewater or substrate is fed (Dold *et al.*, 1980). However, the objective of this section is to determine the proportion of RBCOD in the wastewater with regard to its implication in bulking; modelling of systems fed defined substrate was not an objective of this investigation.

From the work of Gabb (1988) in characterizing the defined substrate it was concluded that the defined substrate had proportions of RBCOD and SBCOD similar to municipal wastewater. As noted in Section A.1, the method of assessment of the results enabling this conclusion to be drawn was somewhat unsound and given the finding that filament proliferation is very different between systems fed defined substrate and those fed municipal sewage, the proportion of RBCOD in the defined

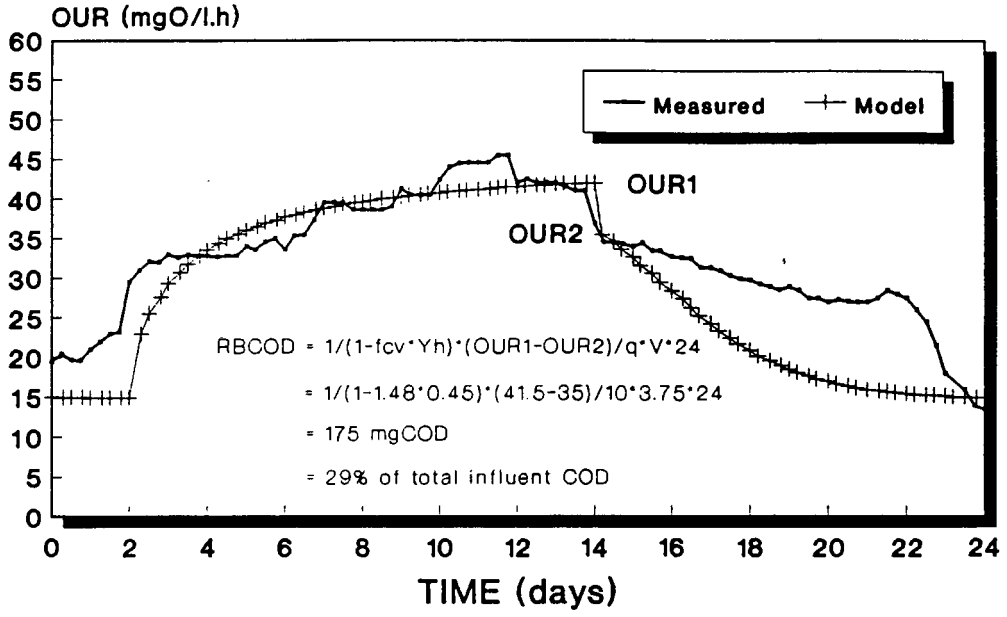


Fig A.19: Oxygen utilization rate (OUR in mgO/g.VSS.h) with time (h) for long sludge age (15 d) single reactor, cyclically loaded (12 h feed on, 12 h feed off) system fed defined substrate composition A.

Table A.33: Theoretically calculated contribution of each of the constituents of the RBCOD suspension.

COD OF CONSTITUENTS OF RBCOD SUSPENSION (From oxidation calculations)	
CONSTITUENTS	COD (mg/d)
Lactose	28
Acetate	59
Succinate	33
Citrate	44
D-Glucose	14
Maltose	148
Glycerol	13
Lactic Acid	52
Ethanol	60
Butanol	37
SUSPENSION	488

Table A.34: Summary of contribution to RBCOD of substrate suspensions as determined by microfiltration, biological square wave, and theoretical means.

RBCOD FRACTION OF SUSPENSIONS OF SUBSTRATE MIXTURE "A" (PERCENTAGE OF TOTAL SUBSTRATE MIXTURE COD)				
METHOD	MICROFILTRATION		BIOLOGICAL SQUARE WAVE	THEORETICAL
SUBSTRATE SUSPENSIONS	Measured (COD <0.45μm)	Calculated (4/5*COD <0.45μm)	Procedure of Ekama *et al.* (1986)	Oxidation calculations
RBCOD	10	7.5	-	8.1
Complex carbohydrates	4	3	-	-
Organic nitrogen	12.5	10	13	-
SUBSTRATE	-	-	30	-

Experimental set–up

Solutions of each of the individual constituents of the Complex Carbohydrates suspension, i.e. starch, dextrin and agar (but not cellulose) were made up to the same concentration as they are in the stock solutions and stored in 2ℓ closed glass flasks at 4° C. A fourth solution comprising a mixture of these solutions in the proportions found in the final substrate feed was stored under the same conditions. Cellulose was not included in the testing programme because of its unbiodegradable nature. The concentrated solutions were tested on a daily basis for a period of 29 days for the fraction of COD passing a 0,45μm filter.

Twice weekly, each of the concentrated solutions (agar, dextrin, starch and mixture) was diluted to the same concentration as is in the final substrate feed mixture, placed in an open 50 mℓ glass flask and fixed to a mechanical shaker for 24 hours to simulate the action of the stirrer in the feed bucket. The 50 mℓ flasks were open to the atmosphere as were the feed buckets in the laboratory investigation. The experiments were terminated after 14 days, this being the longest period for which the concentrated Complex Carbohydrate suspension was stored.

Results

During the 30 day storage experiment, the total COD of each of the samples remained essentially constant; any solubilization of constituents was a transformation of COD > 0,45μm to COD < 0,45μm.

Figure A.20 illustrates the change in COD < 0,45μm (due to solubilization) with time for the complex carbohydrate suspension and the 3 particulate slowly biodegradable constituents, agar, dextrin and starch each stored in concentrated form, as a percentage of the total COD of the complex carbohydrate suspension. The COD fraction < 0,45μm was about 10% of the total COD of the Complex Carbohydrates suspension. Because the Complex Carbohydrates suspension accounts for about 30% of the total COD of the defined substrate, solubilization of Complex Carbohydrates under storage in the concentrated (stock) form is about 3% of the total influent COD of the defined substrate mixture. Note that essentially all solubilization occurs in the first 3 days.

For the diluted, stirred solutions of the Complex Carbohydrate suspension, the changes in COD < 0,45μm with time are shown in Fig A.21. On average, about 14% of the COD of the mixture solubilizes under these conditions, equal to about

4% of the total influent COD of the defined substrate mixture, an additional solubilization of about 1% on the unstirred concentrated suspension as a percentage of the total COD of the substrate.

From the above it can be accepted that the compounds comprising the Complex Carbohydrate suspension did represent a particulate (> 0,45μm) fraction; solubilization of these constituents did not contribute greatly to the RBCOD of the suspension under storage conditions either in the concentrated form (for 1 month), or in the dilute stirred condition (for 24 hrs).

Solubilization of the particulate complex carbohydrate material (> 0,45μm) to soluble (< 0,45μm) material accounted for 4% of the total COD of the defined substrate mixture. Because the < 0,45μm filtration method overestimates the RBCOD content by 25%, the value of COD < 0,45μm (4%) corresponds to a RBCOD content of 3% of the total COD. Adding this amount of < 0,45μm COD (3%) to the < 0,45μm COD measured for the RBCOD suspension (7,5%) (i.e. 10,5% total), leaves 19,5% of the total influent RBCOD measured by the biological method (30%) unaccounted for.

The possibility that some other constituents of the defined substrate, regarded as particulate SBCOD could behave as soluble RBCOD was investigated. Attention was focussed on the Organic Nitrogen suspension.

Determination of the contribution to RBCOD from the Organic Nitrogen suspension
Two methods were used for determination of the RBCOD fraction of the Organic Nitrogen suspension of the defined substrate.

The first method was the biological square wave feeding procedure described earlier. The system was operated with defined substrate which did not include the RBCOD suspension (i.e. substrate feed type C); the only sources of COD were from the Organic Nitrogen and Complex Carbohydrate suspensions. In the defined substrate fed to the square wave system in these tests, the amount of Complex Carbohydrates was increased to accommodate the loss of COD through the omission of the RBCOD suspension (10% of total COD). This was an increase in Complex Carbohydrates from substrate feed type B (see Table A.10). Assuming 14% solubilization of the Complex Carbohydrates suspension, the contribution to COD < 0,45μm is 10% of the total COD of the SBCOD–rich substrate mixture. Given that filtration

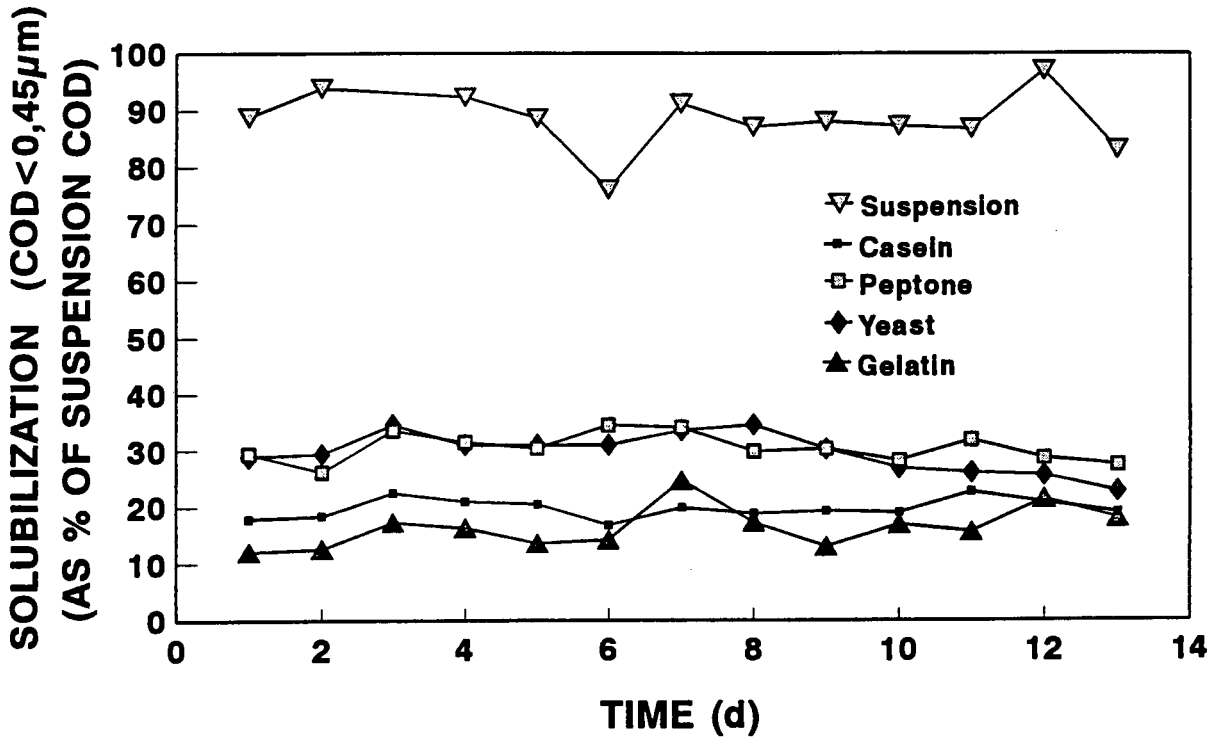


Fig A.22: Solubilization (COD < 0,45µm), with time, for the Organic Nitrogen suspension and its 4 constituents, casein, peptone, yeast, and gelatin, as a percentage of the total COD of the suspension while stored in their concentrated form at 5° C.

APPENDIX B

EXPERIMENTS WITH MUNICIPAL SEWAGE

In the experimental investigation using municipal sewage as influent, three types of system configuration were employed,

(i) single reactor; either intermittently aerated nitrification–denitrification (IAND), continuous aerobic, or continuous anoxic – Systems 5 to 8;

(ii) Two–reactor nitrification–denitrification (2RND) – System 9;

(iii) Multi–reactor nitrification–denitrification biological excess phosphorus removal (NDBEPR) Modified UCT (MUCT) – Systems 10 to 17.

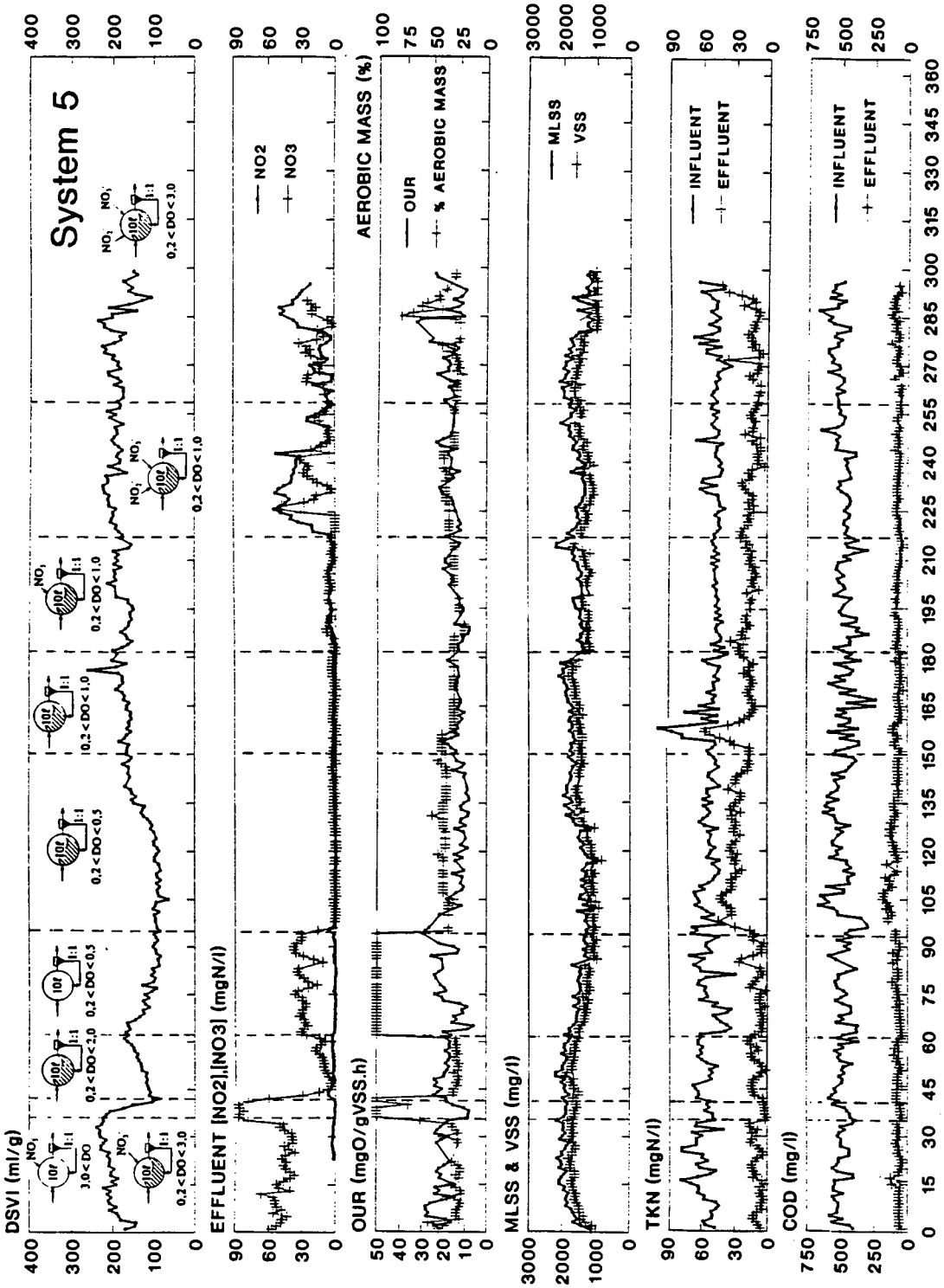

System 5
DSVI (ml/g)
EFFLUENT [NO2],[NO3] (mgN/l)
NO2
NO3
OUR (mgO/gVSS.h)
AEROBIC MASS (%)
OUR
% AEROBIC MASS
MLSS & VSS (mg/l)
MLSS
VSS
TKN (mgN/l)
INFLUENT
EFFLUENT
COD (mg/l)
INFLUENT
EFFLUENT
DAY NO.

Fig B.1

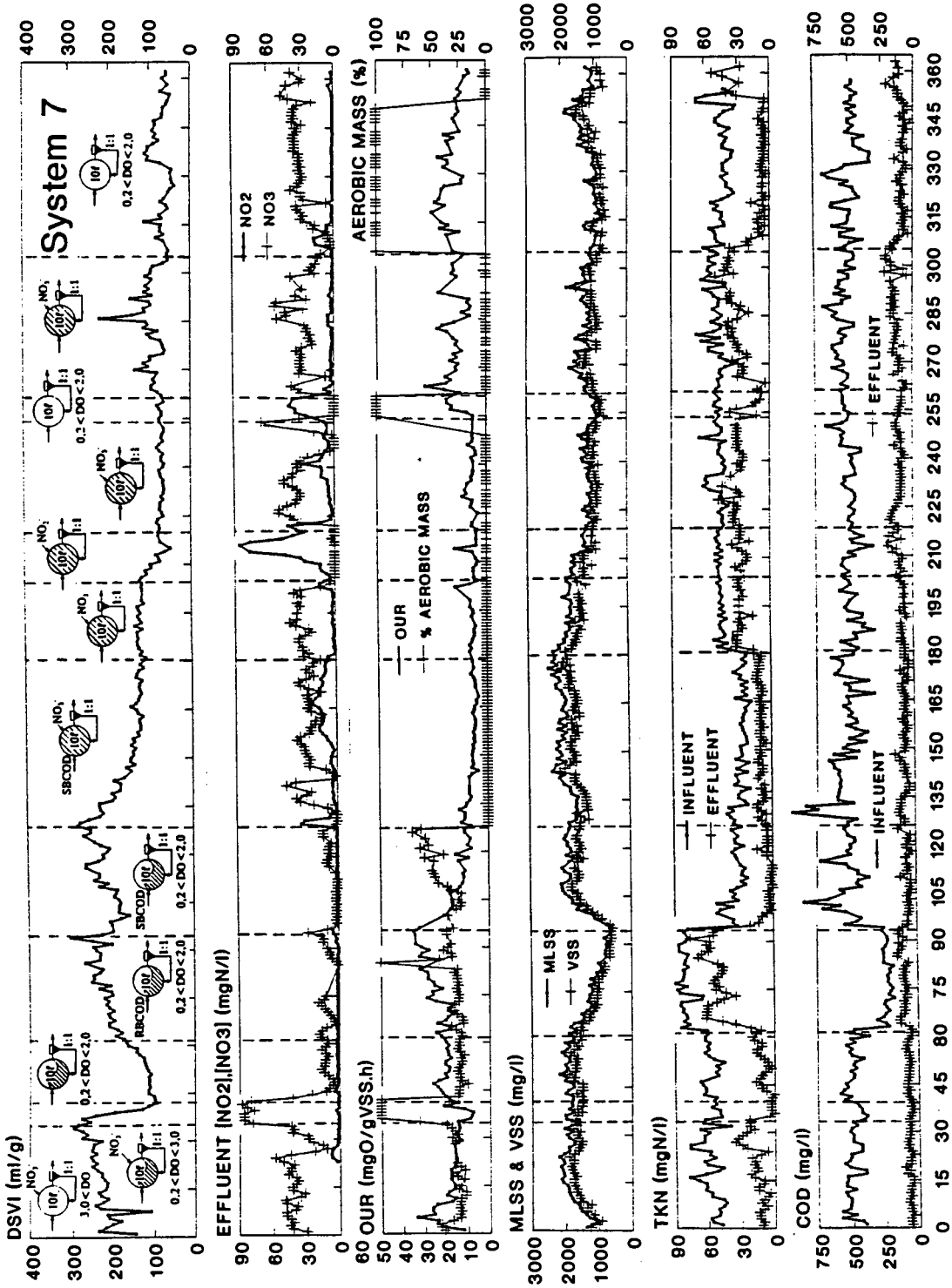

System 7
DSVI (ml/g)
EFFLUENT [NO2],[NO3] (mgN/l)
NO2
NO3
OUR (mgO/gVSS.h)
AEROBIC MASS (%)
OUR
% AEROBIC MASS
MLSS & VSS (mg/l)
MLSS
VSS
TKN (mgN/l)
INFLUENT
EFFLUENT
COD (mg/l)
INFLUENT
EFFLUENT
DAY NO.

Fig B.3

Table B.1

SYSTEM 5 :		CHANGES IN OPERATING CONDITIONS
Period (Days)	Operating conditions	Reasons for changes in operating conditions: Comments on system response
1 - 35	Intermittent aeration - very high DO ($0,2 < DO < 3,0$ mgO/ℓ) 30-35% aerobic	To determine if intermittent aeration induces AA filament proliferation: The DSVI increased rapidly, from ≈150 to ≈225 mℓ/g; a rate similar to the increase in DSVI during the same period in Systems 6 - 8 and *M. parvicella* was the dominant filament.
36 - 42	Continuous aeration ($DO > 3,0$ mgO/ℓ)	To determine if continuous aeration reduces AA filament proliferation: The DSVI decreased rapidly from ≈225 to <100 mℓ/g; a rate similar to the decrease in DSVI during the same period in Systems 6 - 8.
43 - 61	Intermittent aeration - high DO ($0,2 < DO < 2,0$ mgO/ℓ) 30-35% aerobic	To increase the DSVI to above 150 mℓ/g: The DSVI increased from <100 to ≈165 mℓ/g and *M. parvicella* was dominant.
62 - 94	Continuous aeration - low DO ($0,2 < DO < 0,5$ mgO/ℓ)	To determine if AA filaments proliferate in the low DO period ($0,2 < DO < 0,5$) of IAND systems: The DSVI decreased from ≈265 mℓ/g to ≈100 mℓ/g. Note that the concentration of NO_2^- in the effluent (<1,0 mgN/ℓ) is considerably less than the concentration of NO_3^- (≈30 mgN/ℓ)
95 - 150	Intermittent aeration - low DO ($0,2 < DO < 0,5$ mgO/ℓ) 30-35% aerobic	To determine if AA filaments can proliferate under intermittent aeration conditions if the aerobic period of the low DO period is DO ($0,2 < DO < 0,5$): The DSVI did not increase until Day 120, then increased to a constant value ≈160 mℓ/g by Day 150. The dominant filament was *H. hydrossis*. Note that in contrast to the continuous low DO conditions, the concentrations of NO_2^- (1,5 mgN/ℓ) and NO_3^- (1,5 mgN/ℓ) are similar. This aspect is discussed in Appendix E in dealing with nitrification.
151 -181	Intermittent aeration - intermediate DO ($0,2 < DO < 1,0$ mgO/ℓ) 30-35% aerobic	To determine if filaments proliferate more when the DO in the aerobic period is increased: The DSVI increased from ≈160 mℓ/g to ≈200 mℓ/g and type 0092 was dominant.
182 - 217	Intermittent aeration - intermediate DO ($0,2 < DO < 1,0$ mgO/ℓ) 30-35% aerobic Continuous NO_3^- addition	To determine if high concentrations of NO_3^- increase filament proliferation: The DSVI was not constant but remained between 150 and 200 mℓ/g and filament types 0092 and 0041 were both dominant for some of the time. Note that although NO_3^- was being added, the proportion of NO_2^- (≈4 mgN/ℓ) to NO_3^- (≈4 mgN/ℓ) did not change.
218 - 258	Intermittent aeration - intermediate DO ($0,2 < DO < 1,0$ mgO/ℓ) 30-35% aerobic Continuous NO_2^- and NO_3^- addition	To determine the effect of high concentrations of NO_3^- (>10 mgN/ℓ): The DSVI fluctuated between 180 and 220 mℓ/g. The dominant filament was *M. parvicella*.
259 - 300	Intermittent aeration - high DO ($2,0 < DO < 3,0$ mgO/ℓ) 30-35% aerobic Continuous NO_3^- and NO_2^- addition	To determine if increasing the DO in the aerobic period ($2,0 < DO < 3,0$) under intermittent aeration conditions results in filament proliferation: The DSVI increased to ≈225 mℓ/g by Day 276 and then decreased to ≈150 mℓ/g by Day 300. The increase in DSVI resulted from an increase in percent aerobic fraction caused a loss of sludge in the settler. This aspect is discussed in greater detail in chapter 3.

Table B.3

SYSTEM 7 :	CHANGES IN OPERATING CONDITIONS	
Period (Days)	Operating conditions	Reasons for changes in operating conditions: Comments on system response
1 - 35	Intermittent aeration - very high DO (0,2<DO<3,0) 30-35% aerobic	To determine if intermittent aeration induces filament proliferation: The DSVI increased steadily from ≈ 200 to ≈ 290 ml/g; (compare Systems 5, 6 and 8). Filament type 021N was dominant. Effluent NO_{3-5}^- (≈ 45 mgN/ℓ).
36 - 42	Continuous aeration - very high DO (DO>3,0 mgO/ℓ)	To determine if continuous aeration reduces filament proliferation: The DSVI decreased rapidly from ≈ 290 ml/g to <100 ml/g; a rate similar to the decrease in DSVI during the same period in Systems 5, 6 and 8. Note the increase in effluent NO_{3-5}^- (≈ 85 mgN/ℓ) due to more complete nitrification of NH_4^+. Note that COD mass balance (100%) is better than the previous intermittent aeration period (95%) (see Fig. 3.11 & Table B.12).
43 - 62	Intermittent aeration - very high DO (0,2<DO<2,0) 30-35% aerobic	Note that the increase in effluent NO_{3-5}^- (≈ 85 mgN/ℓ) compared to intermittent aeration. Note that the COD mass balance (93%) is better than the previous intermittent aeration period (see Fig. 3.11 & Table B.12) To increase the DSVI to above 150 ml/g: The DSVI increased from <100 to ≈ 165 ml/g (compare with Systems 5, 7 and 8). Filament type 021N was dominant.
63 - 94	Intermittent aeration - high DO (0,2<DO<2,0) 30-35% aerobic RBCOD substrate	To determine filaments proliferate with RBCOD: The DSVI increased to 250 ml/g (compare with System 8, Days 95-127). The dominant filament was type 021N. The high influent NH_4^+(72 mgN/ℓ) was a result of NH_4^+ in the soluble fraction of sewage, effluent NO_2^- and NO_3^- were ≈ 1 and ≈ 10 mgN/ℓ respectively.
95 - 127	Intermittent aeration - high DO (0,2<DO<2,0) 30-35% aerobic SBCOD substrate	To determine if filaments proliferate with SBCOD: The DSVI decreased initially, then increased to ≈ 250 ml/g (compare with System 8, Days 63-94). The dominant filament was type 021N. The effluent NO_2^- (≈ 0,7 mgN/ℓ) and NO_3^- (≈ 6 mgN/ℓ) were not very different even through the influent NH_4^+ (17 mgN/ℓ) was less than for RBCOD as substrate.
128 - 180	Continuous anoxic Continuous NO_3^- addition SBCOD substrate	To determine if filaments proliferate under anoxic conditions: The DSVI decreased to 130 ml/g. The dominant filament was initially *H. hydrossis*, and then type 0092. The effluent NO_2^- concentration increased from 1 to ≈ 10 mgN/ℓ as the DSVI decreased.
181 - 202	Continuous anoxic Continuous NO_3^- addition	Change from SBCOD to non-fractionated average sewage: The DSVI remained constant at ≈ 130 ml/g.
203 - 221	Continuous anoxic Continuous NO_2^- addition	To determine if filaments can proliferate with NO_2^- as electron acceptor under anoxic conditions: The DSVI decreased to ≈ 75 ml/g.
222 - 254	Continuous anoxic Continuous NO_3^- addition	To determine if changing electron acceptor to NO_3^- results in increase in DSVI: The DSVI remained constant at ≈ 75 ml/g.
255 - 261 262 - 305 306 - 352 353 - 363	Continuous aerobic - Continuous anoxic - Continuous aerobic - Continuous anoxic -	Experiments to determine the extent of synthesis of aerobic and denitrifying enzymes under aerobic and anoxic conditions (see chapter 6): DSVI remained < 100 ml/g. Note that the COD mass balance for the aerobic period, Days 335-352 (90%) is better than for periods of intermittent aeration (see Table B.12).

Table B.5

Day No. System 5	Filament abundance	Dominant	Secondary	Tertiary	Remarks
7	abundant	021N	*M.parvicella*, 0092	0041, 1851, 0961	
38	some-common	*M.parvicella*	0092	*Thiothrix* sp.	•chains of large cocci
69	common-very common	*M.parvicella*	0092	*H.hydrossis*, 0961, 0041, 021N	
96	few-some	*H.hydrossis*	0041	*M.parvicella*, 0961	•*Fungus* sp. present
129	common-very common	0092	021N	*M.parvicella, Beggiatoa* sp., 0841, *H.hydrossis*	
152	common-very common	0092	*H.hydrossis*	*Beggiatoa* sp., 0961, *M.parvicella*, 0041	
187	very common	0041	0092	0961, *M.parvicella, Beggiatoa* sp., *H.hydrossis, N.limicola I*	
227	common-very common	0041	0092	021N, *H.hydrossis, M.parvicella*	•chains of cocci
257	very common-abundant	*M.parvicella*	0092	0041, *H.hydrossis*, 021N	•chains of cocci
298	very common-abundant	*M.parvicella*	0092	*Beggiatoa* sp., *Flexibacter*, 0041, 1851, 021N	•attached ciliates common

Table B.7

Day No. System 7	Filament abundance	Dominant	Secondary	Tertiary	Remarks
7	abundant	021N	*M.parvicella*, 0092	0041, 1851, 0961	
38	common-very common	021N	*M.parvicella*	0092, 0041	
69	abundant	021N	*M.parvicella*	*H.hydrossis*, 0041, 0092	• Attached cilates common
96	some-common	021N	0041	*H.hydrossis*, *M.parvicella*, 0092	• Fungus sp. present
129	very common-abundant	021N	*H.hydrossis*, 0092	0041, *M.parvicella*	
152	very common	*H.hydrossis*	0092	*M.parvicella*, 0041, 021N	
187	very common	0092	021N	*H. hydrossis*, 0041	
227	few-some	0092	-	*H.hydrossis*, 0041, 1851	• Amoeba fairly common
257	some	021N	-	*S.natans*, 0092, 0041, 0803, *H.hydrossis*	
298	some-common	0092	*M.parvicella* (broken)	*Beggiatoa* sp., *H.hydrossis*	• Free-swimming ciliates common
336	some-common	*H.hydrossis*	-	0041, 0092, 021N, 0803	• Finger-like zooglea

Table B.9

THE EFFECT OF VARIOUS CONDITIONS ON FILAMENTOUS ORGANISMS DEVELOPED ON MUNICIPAL SEWAGE			
ORGANISM TYPE	**Conditions which promote growth**	**Conditions which reduce growth**	**Conditions which sustain growth**
Microthrix parvicella	• Intermittent aeration - high DO (DO > 2,0 mgO/ℓ) + NO_3^- concentration > 1 mgN/ℓ + SBCOD as substrate	• Intermittent aeration - low DO (DO < 2,0 mgO/ℓ) + NO_3^- concentration < 1 mgN/ℓ • Intermittent aeration - high DO (DO > 3,0 mgO/ℓ) + RBCOD substrate • Continuous aeration - high DO (DO > 3,0 mgO/ℓ) • Continuous aeration - low DO (02 < DO < 0,5 mgO/ℓ)	
0092	• Intermittent aeration - low DO (0,2 < DO < 0,5) • Intermittent aeration - high DO (DO > 2,0 mgO/ℓ) + anaerobic zone in system (not immediate to aerobic reactor)	• Continuous aeration - high DO (DO > 2,0 mgO/ℓ)	• Intermittent aeration - high DO + low NO_3^- concentrations • Continuous aeration - low DO (0,2 < DO < 0,5)
Haliscomenobacter Hydrossis	• Intermittent aeration - high DO + RBCOD (DO > 2,0 mgO/ℓ)	• Continuous aeration - high DO (DO > 2,0 mgO/ℓ) • Continuous anoxic	
0041	• Change in aeration pattern: intermittent (high DO) - continuous (low DO) intermittent (low DO) - intermittent (intermediate DO)		• Continuous aeration - high D (0,5 < DO < 2,0) • RBCOD under aerobic conditions
021N	• Septic sewage under: intermittent high DO (DO > 2,0 mgO/ℓ)	• Continuous high DO (DO > 3,0 mgO/ℓ) + removal of septic sewage	

Table B.11

COD & NITROGEN Balance: SYSTEM 6

Q =	10	l/d
Vp =	10	l
Rs =	15	d
w =	667	ml
fn =	0.1	mgN/mgVSS
fcv =	1.48	mgCOD/mgVSS

MSti: influent COD
MSte: effluent COD
Xv: volatile solids
MZw: sludge wasted
MOc: carbonac.O2 demand
MNti: influent nitrogen
MNte: effluent nitrogen
MNw: waste sludge nitrogen
MNoxi: nitrate/nitrite added

MNoxd: nitrate denitrified
MNoxn: nitrate generated by nitrification
%Aer: Percentage aerobic
MOt: total O2 consumed
MOn: nitrification O2 requirement
MOh: heterotrophic O2 requirement
MOd: denitrification O2 equivalent
MNoxe: effluent nitrate

Steady State Period	Day-Day		MSti mg/d	MSte mg/d	Xv mg/l	MZw mg/d	MOc mgO/d	COD Mass Balance	Steady State Period
1	1	35	4940	695	1685	1662	2141	91	1
2	36	42	4884	607	1699	1676	2503	98	2
3	43	60	4928	710	1756	1732	1311	76	3
4	61	80	4596	662	1852	1827	1289	82	4
5	81	85	4477	870	1771	1747	1547	93	5
6	86	94	4784	964	1353	1335	1095	71	6
7	95	120	4849	748	1468	1449	2355	94	7
8	121	153	5087	814	1312	1295	3123	103	8
9	154	188	4392	734	1412	1393	1493	82	9
10	189	196	4500	496	1520	1499	1943	88	10
11	197	213	4767	421	1532	1512	1920	81	11
12	214	225	4545	470	1271	1254	3041	105	12
13	226	265	4953	593	1038	1024	2454	82	13

Steady State Period	Day-Day		MNti	MNte	MNw	MNoxi	MNoxe	MNoxd	MNoxn	Nitrogen Mass Balance	Steady State Period
1	1	35	583	110	112	577	443	494	360	100	1
2	36	42	542	31	113	414	835	-23	397	100	2
3	43	60	580	89	117	0	149	225	374	100	3
4	61	80	475	214	123	0	43	94	138	100	4
5	81	85	539	311	118	0	11	99	110	100	5
6	86	94	566	348	90	0	18	110	128	100	6
7	95	120	548	76	98	0	253	121	374	100	7
8	121	153	518	91	87	0	196	143	339	100	8
9	154	188	532	67	94	0	178	193	371	100	9
10	189	196	446	67	101	0	202	76	278	100	10
11	197	213	465	73	102	0	22	268	290	100	11
12	214	225	456	55	85	0	242	74	316	100	12
13	226	265	475	60	69	0	314	31	345	100	13

Steady State Period	Day-Day		OUR mgO/l.h	%Aer	MOt mgO/d	MOn mgO/d	MOh mgO/d	MOd mgO/d	Steady State Period
1	1	35	33.4	30	2376	1647	729	1412	1
2	36	42	19.0	96	4384	1814	2570	-67	2
3	43	60	34.3	29	2376	1708	668	643	3
4	61	80	20.0	34	1648	629	1019	270	4
5	81	85	21.0	35	1767	505	1263	284	5
6	86	94	13.5	42	1365	584	781	314	6
7	95	120	15.5	100	3718	1709	2009	346	7
8	121	153	17.8	100	4264	1548	2715	408	8
9	154	188	26.1	42	2635	1694	941	552	9
10	189	196	29.7	42	2995	1270	1726	217	10
11	197	213	31.9	32	2480	1325	1155	765	11
12	214	225	17.8	100	4272	1443	2829	213	12
13	226	265	16.4	100	3943	1579	2364	90	13

Table B.13

COD & NITROGEN Balance: SYSTEM 8

Q = 10 l/d
Vp = 10l (1-97); 15l (96-226)
Rs = 15 d
w = 667ml (10l); 1000ml (15l)
fn = 0.1 mgN/mgVSS
fcv = 1.48 mgCOD/mgVSS

MSti: influent COD
MSte: effluent COD
Xv: volatile solids
MZw: sludge wasted
MOc: carbonac.O2 demand
MNti: influent nitrogen
MNte: effluent nitrogen
MNw: waste sludge nitrogen
MNoxi: nitrate/nitrite added
MNoxd: nitrate denitrified
MNoxm: nitrate generated by nitrification
%Aer: Percentage aerobic
MOt: total O2 consumed
MOn: nitrification O2 requirement
MOh: heterotrophic O2 requirement
MOd: denitrification O2 equivalent
MNoxe: effluent nitrate

Steady State Period	Day-Day	MSti mg/d	MSte mg/d	Xv mg/l	MZw mg/d	MOc mgO/d	COD Mass Balance	Steady State Period
1	1 35	4940	677	1612	1590	2207	91	1
2	36 42	4884	650	1593	1572	2633	99	2
3	43 62	4933	712	1795	1771	1525	81	3
4	63 94	5465	564	1880	1855	1864	78	4
5	95 119	3852	1979	445	658	1248	101	5
6	120 127	5065	2633	444	657	2033	105	6
7	128 135	5643	2288	492	728	1650	83	7
8	136 162	4489	1976	499	738	1250	88	8
9	163 193	3291	1605	388	575	848	92	9
10	194 226	2904	1763	291	431	1967	143	10

Steady State Period	Day-Day	MNti	MNte	MNw	MNoxi	MNoxe	MNoxd	MNoxm	Nitrogen Mass Balance	Steady State Period
1	1 35	583	150	107	440	429	336	325	100	1
2	36 42	542	29	106	374	754	26	406	100	2
3	43 62	582	106	120	0	149	208	357	100	3
4	63 94	381	49	125	0	43	164	207	100	4
5	95 119	1524	792	30	0	391	312	703	100	5
6	120 127	1779	1276	30	0	36	437	473	100	6
7	128 135	1718	1089	33	510	701	405	596	100	7
8	136 162	1659	981	33	544	797	392	645	100	8
9	163 193	1451	829	26	532	999	129	596	100	9
10	194 226	1452	734	19	565	676	587	698	100	10

Steady State Period	Day-Day	OUR mgO/l.h	%Aer	MOt mgO/d	MOn mgO/d	MOh mgO/d	MOd mgO/d	Steady State Period
1	1 35	28.6	40	2732	1485	1247	960	1
2	36 42	18.4	100	4413	1856	2557	76	2
3	43 62	33.7	32	2561	1630	931	594	3
4	63 94	22.5	41	2223	827	1396	468	4
5	95 119	17.2	51	3167	2811	355	893	5
6	120 127	16.3	46	2676	1893	783	1250	6
7	128 135	15.6	51	2876	2385	491	1159	7
8	136 162	19.6	38	2709	2579	129	1121	8
9	163 193	23.3	34	2862	2384	478	370	9
10	194 226	27.9	31	3082	2793	289	1679	10

f_n = nitrogen fraction of VSS
= 0,10 mgN/mgVSS
= $M\Delta X_v$ = mass of sludge wasted/d (mgVSS/d)

(iv) $(NO_2^- + NO_3^-)$ denitrified: MN_{oxd}

$MN_{oxd} = MN_{oxi} + (MN_{ti} - MN_{te} - MN_w) - MN_{oxe}$

$\%N \text{ balance} = 100 \cdot (MN_{te} + MN_{oxe} + MN_w + MN_{oxd})/MN_{ti} + MN_{oxi}$

Example calculation

$MN_{ti} = 10\ \ell/d \cdot 58{,}3\ mgN/\ell = 583\ mgN/d$

$MN_{oxi} = 541\ mgN/d$

$MN_{te} = 10\ \ell/d \cdot 8{,}8\ mgN/\ell = 88\ mgN/d$

$MN_{oxe} = 10\ \ell/d \cdot 47{,}9\ mgN/\ell = 479\ mgN/d$

for MN_w : $M\Delta X_v = 1617\ mgVSS/\ell \cdot 0{,}667\ \ell/d = 1079\ mgVSS/d$

$MN_w = 0{,}10\ mgN/mgVSS \cdot 1079\ mgVSS/d = 108\ mgVSS/d$

$MN_{oxd} = 541 + \{583 - 88 - 108\} - 479 = 449\ mgN/d$

$\%\ N \text{ balance} = 100 \cdot (88 + 479 + 108 + 449)/(583 + 541) = 100$

COD mass balance

COD in:

(i) COD in influent: MS_{ti}

$MS_{ti} = Q \cdot S_{ti}$

where

S_{ti} = average of daily effluent COD concentration

COD out:

(i) COD utilized under aerobic conditions (in O_2 units): $M(O_h)$

$MO_h = MO_t - MO_n$

where

MO_t = mass of oxygen utilized in aerobic period

MO_n = mass of oxygen for nitrification

$MO_t = (OUR \cdot V_t \cdot 24 \cdot p_a)$

where

MO_h = (2537–1769) mgO/d = 768 mgO/d

MO_d = 2,86 mgO/mgN · 449 mgN/d = 1284

MZ_w = 1,48 mgCOD/mgVSS · 1079 mgVSS/d = 1597 mgCOD/d

MS_{te} = 10 ℓ/d · 63,9 mgCOD/ℓ = 639 mgCOD/d

% COD balance = 100 (768 + 1284 + 1597 + 639)/4940 = 87

(e.g. MO_t; 2537 and 2520 mgO/d) are a consequence of working with values rounded off to the nearest unit as opposed to the 4 decimal places in the computer programme used for calculation of the tabulated data.

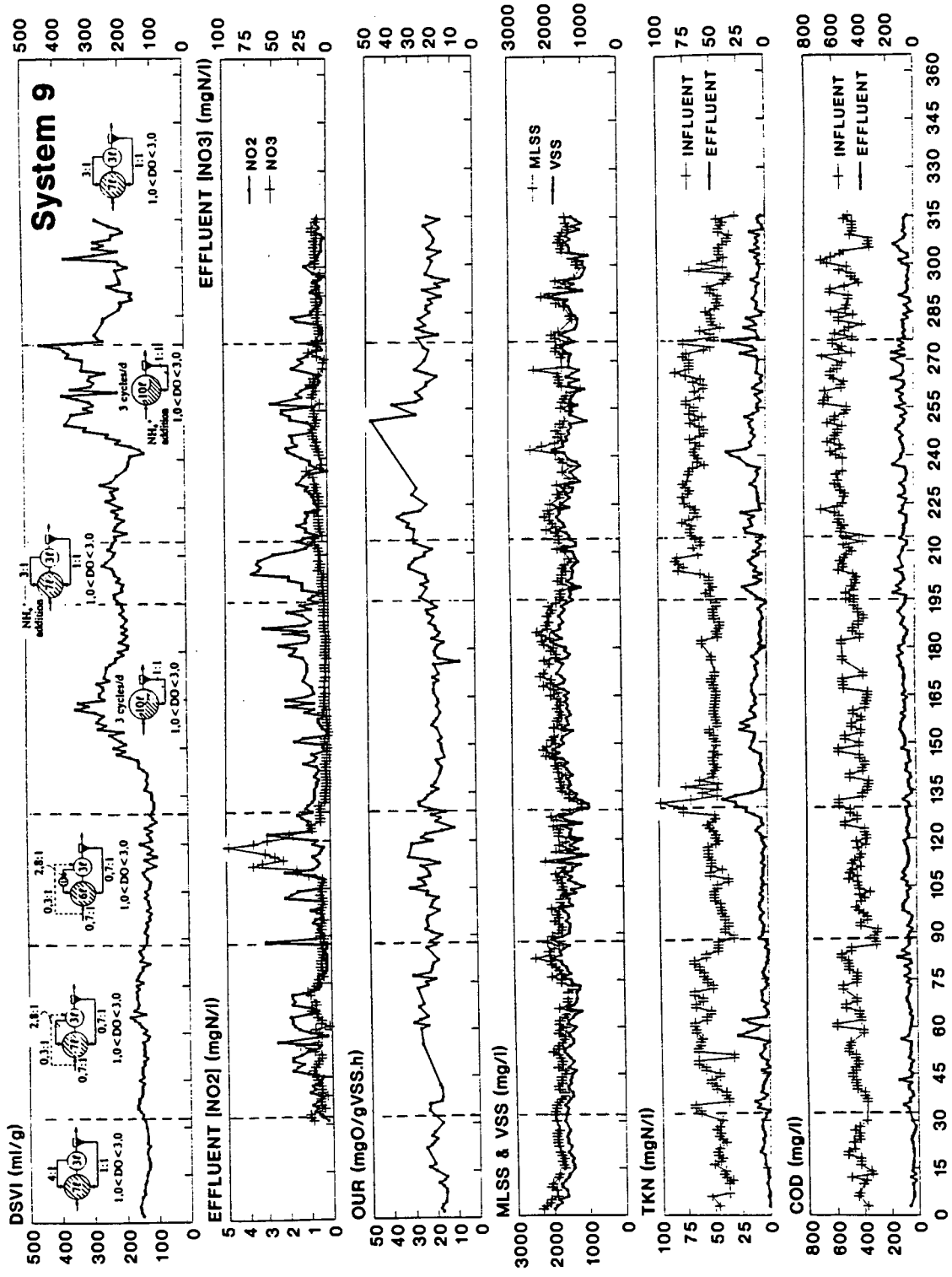

System 9
DSVI (ml/g)
EFFLUENT [NO2] (mgN/l)
EFFLUENT [NO3] (mgN/l)
NO2
NO3
OUR (mgO/gVSS.h)
MLSS & VSS (mg/l)
MLSS
VSS
TKN (mgN/l)
INFLUENT
EFFLUENT
COD (mg/l)
INFLUENT
EFFLUENT
DAY NO.

Fig B.5

Table B.15

Day No. System 9	Filament abundance	Dominant	Secondary	Tertiary	Remarks
11	very common	021N	0092	*H.hydrossis*, *M.parvicella*, *Nocardia*, 0041, *Aeolosoma* sp.	• Chains of cocci
42	very common-abundant	021N	0092	*M.parvicella*, 0041, 0092	
69	common	021N	*M.parvicella*	*Beggiatoa* sp., 0041, 0092	
102	abundant	0041	*M.parvicella*	*H.hydrossis*, 0092, 021N, Flexibacter	• Finger-like zooglea
125	very common	0041	0092	*M.parvicella*, *H.hydrossis*, 1851	• Diatoms common • Free-swimming ciliates common • Finger-like zooglea
160	very common-abundant	*H.hydrossis*	0041, 0092	*Beggiatoa*, 021N, *M.parvicella*	
200	very common	0041	-	Flexibacter, 0092, *H.hydrossis*, *M.parvicella*, 0803	• Diatoms common
230	some-common	0041	-	*M.parvicella*, *S.natans*, 1851, 0092	
271	common-very common	0041	*M.parvicella*	021N, *S.natans*, 0092	
309	very common	0803	0041	*H. hydrossis*, 0092	• Spirochaetes abundant

to 13, combined NO_2^- and NO_3^- concentrations were measured, and for Systems 14 to 17 individual NO_2^- and NO_3^- concentrations were measured. The nitrogen mass balance example is for System 14, steady state period 2.

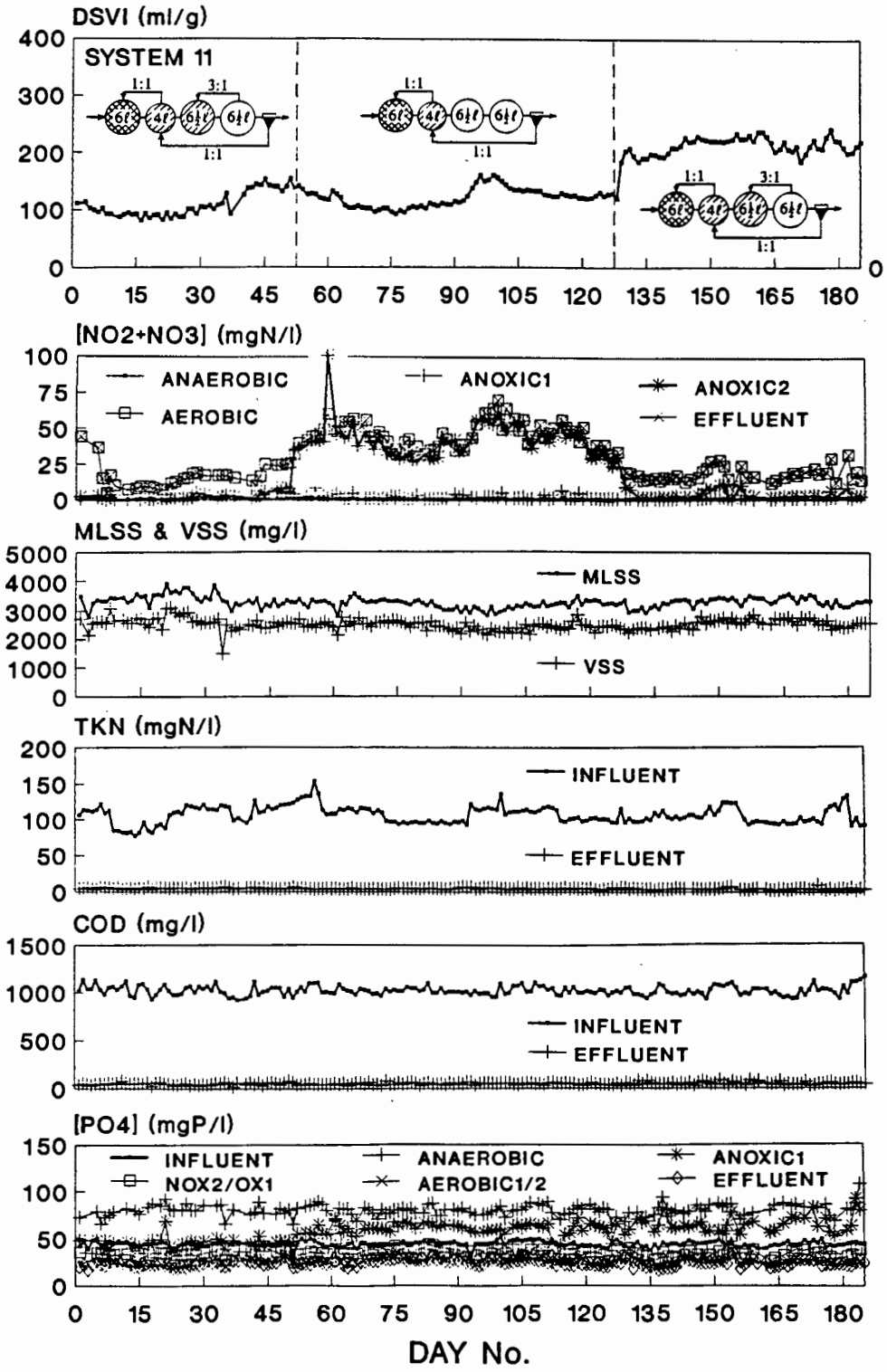

DSVI (ml/g)
SYSTEM 11
1:1
3:1
1:1
[NO2+NO3] (mgN/l)
ANAEROBIC
AEROBIC
ANOXIC1
ANOXIC2
EFFLUENT
MLSS & VSS (mg/l)
MLSS
VSS
TKN (mgN/l)
INFLUENT
EFFLUENT
COD (mg/l)
INFLUENT
EFFLUENT
[PO4] (mgP/l)
INFLUENT
NOX2/OX1
ANAEROBIC
AEROBIC1/2
ANOXIC1
EFFLUENT
0 15 30 45 60 75 90 105 120 135 150 165 180
DAY No.

Fig B.7

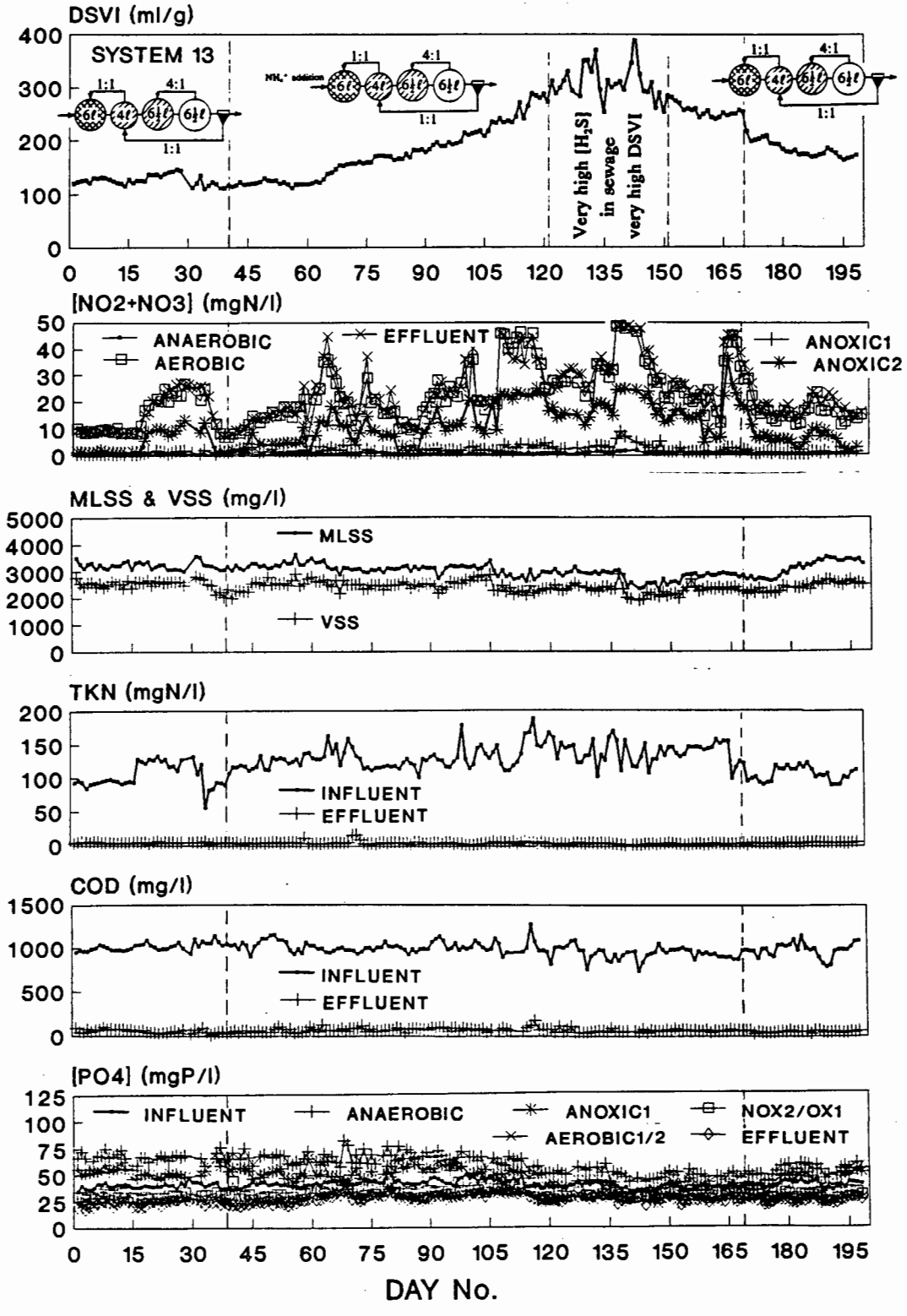

DSVI (ml/g)
SYSTEM 13
1:1
4:1
1:1
Very high [H2S] in sewage very high DSVI
[NO2+NO3] (mgN/l)
ANAEROBIC
AEROBIC
EFFLUENT
ANOXIC1
ANOXIC2
MLSS & VSS (mg/l)
MLSS
VSS
TKN (mgN/l)
INFLUENT
EFFLUENT
COD (mg/l)
INFLUENT
EFFLUENT
[PO4] (mgP/l)
INFLUENT
ANAEROBIC
ANOXIC1
NOX2/OX1
AEROBIC1/2
EFFLUENT
DAY No.

Fig B.9

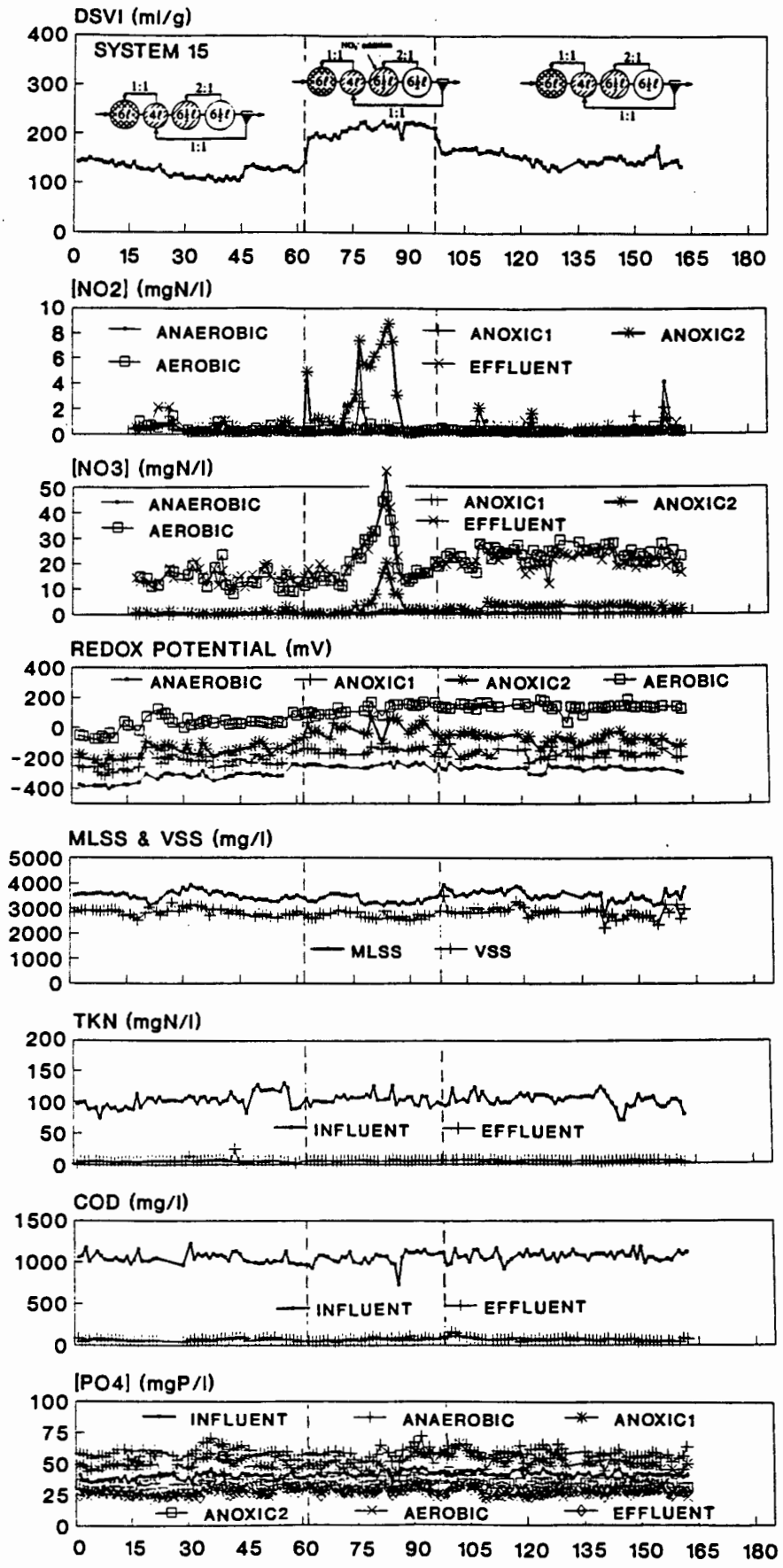

DSVI (ml/g)
SYSTEM 15
[NO2] (mgN/l)
ANAEROBIC
AEROBIC
ANOXIC1
EFFLUENT
ANOXIC2
[NO3] (mgN/l)
REDOX POTENTIAL (mV)
MLSS & VSS (mg/l)
MLSS
VSS
TKN (mgN/l)
INFLUENT
COD (mg/l)
[PO4] (mgP/l)
DAY No.

Fig B.11

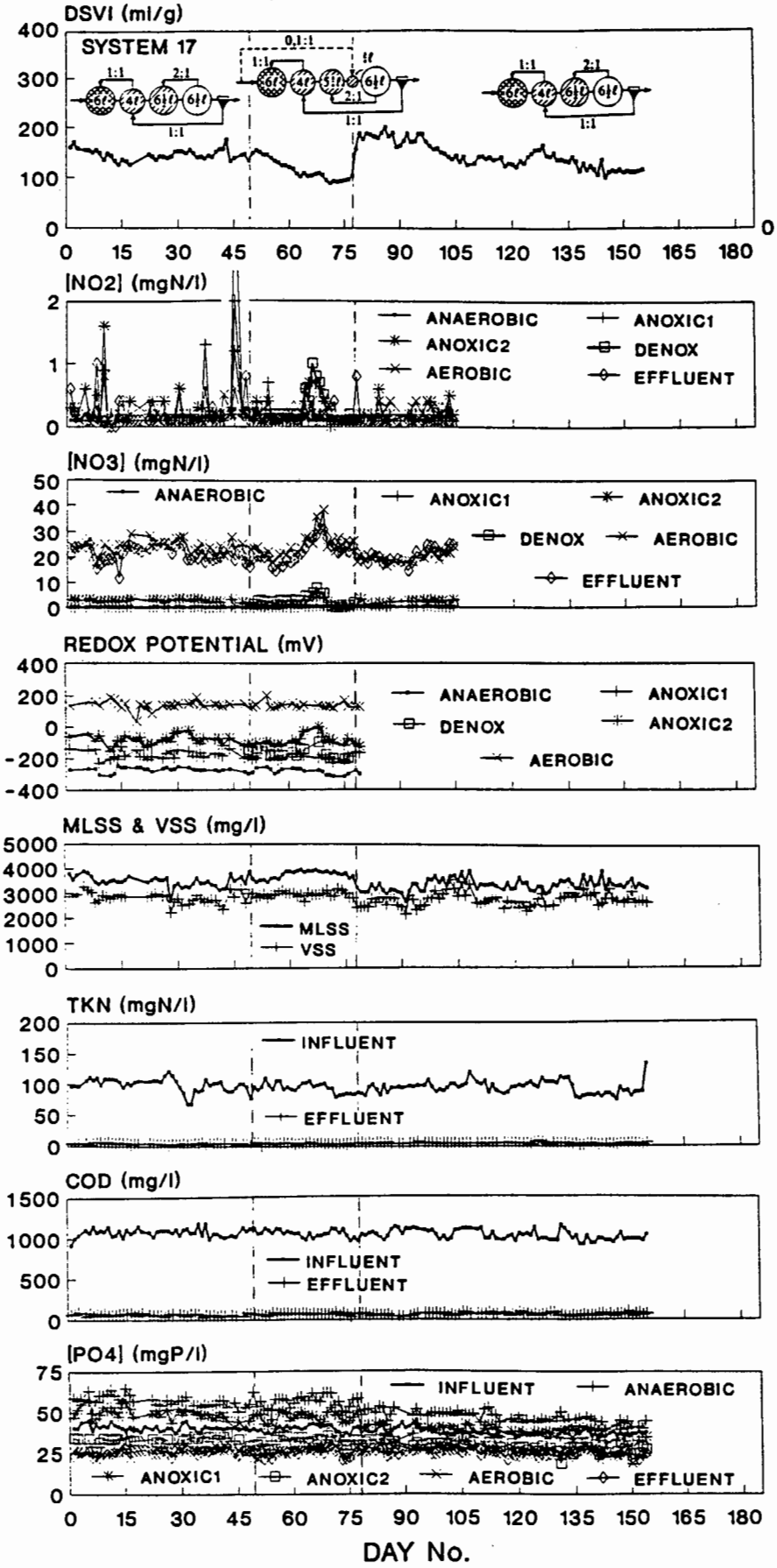

DSVI (ml/g)
SYSTEM 17
[NO2] (mgN/l)
ANAEROBIC
ANOXIC1
ANOXIC2
DENOX
AEROBIC
EFFLUENT
[NO3] (mgN/l)
REDOX POTENTIAL (mV)
MLSS & VSS (mg/l)
MLSS
VSS
TKN (mgN/l)
INFLUENT
COD (mg/l)
[PO4] (mgP/l)
DAY No.

Fig B.13

Table B.17

SYSTEM 12 :	CHANGES IN OPERATING CONDITIONS	
Period (Days)	Operating conditions	Reasons for changes in operating conditions: Comments on system response
1 - 40	MUCT configuration 33% aerated Low TKN/COD (0,08-0,10)	This configuration used to establish a sludge containing filament type 0092 and as a complement to System 13 (Days 1-40): DSVI constant at 100-150 $m\ell/g$; dominant filament is type 0092. Note the increase in DSVI (Days 21-28) is paralleled by an increase in NO_{x+3}^- concentration in the 2nd anoxic and aerobic reactors and effluent (see Fig. 3.17).
41 - 121	MUCT configuration 33% aerated Low TKN/COD (0,08-0,10)	Configuration same as for Days 1-40 as a complement to System 13 (Days 41-121): DSVI remained between 100 and 150 $m\ell/g$; dominant filament is type 0092 and type 021N secondary. Note the gradual decrease in VSS with increase in DSVI (see Fig. 3.17).
122 - 150	MUCT configuration 33% aerated Low TKN/COD (0,08-0,10)	No change in configuration: DSVI increased rapidly as a consequence of proliferation of 021N due to the high concentration of H_2S in the sewage. System cleaned - tubing disinfected.
151 - 170	MUCT configuration 33% aerated Low TKN/COD (0,08-0,10)	No change in configuration: DSVI decreased from ≈ 140 to < 100 $m\ell/g$; dominant filament is type 0092. Note the decrease in concentration of NO_{x+3}^- in the second anoxic reactor.
171 - 198	MUCT configuration 33% aerated High TKN/COD (0,12-0,14) (from NH_4^+ addition to influent)	To determine effect of NO_{x+3}^- produced from nitrification of NH_4^+ on filament proliferation: DSVI increased from < 100 to ≈ 240 $m\ell/g$; dominant filament is type 0092. Note the decrease in VSS with increase in DSVI (see Fig. 3.17).
	SYSTEM 13	
1 - 40	MUCT configuration 33% aerated	This configuration used to establish a sludge containing filament type 0092 and as a complement to System 12 (Days 1-40): DSVI constant at 100-150 $m\ell/g$; dominant filament type 0092.
41 - 121	MUCT configuration 33% aerated High TKN/COD (0,12-0,14 mgN/mgCOD) (from NH_4^+ addition to influent)	To determine effect of NO_{x+3}^- produced from nitrification of NH4$^+$ on filament proliferation: DSVI increased from ≈ 120 to ≈ 280 $m\ell/g$. The dominant filament was type 0092 initially and then type 021N dominated and type 0092 was secondary.
122 - 150	MUCT configuration 33% aerated High TKN/COD (0,12-0,14 mgN/mgCOD) (from NH_4^+ addition to influent)	No change in configuration: DSVI increased rapidly as a consequence of proliferation of 021N due to the high concentration of H_2S in the sewage. Note the decrease in VSS (see Fig. 3.18). System cleaned - tubing disinfected.
151 - 170	MUCT configuration 33% aerated High TKN/COD (0,12-0,14 mgN/mgCOD) (from NH_4^+ addition to influent)	No change in configuration: DSVI decreased slightly from ≈ 280 to ≈ 250 $m\ell/g$; dominant filament type 0092, secondary 021N.
171 - 198	MUCT configuration 33% aerated Low TKN/COD (0,08-0,10 mgN/mgCOD)	To determine effect of low NO_{x+3}^- concentration on filament proliferation and as a complement to System 12 (Days 171-198): DSVI decreased from ≈ 250 to ≈ 165 $m\ell/g$; dominant filament type 021N, type 0092 secondary.

Summary

- Low influent TKN/COD ratios and associated 2 anoxic reactor low NO_{x+3}^- concentrations, result in lower DSV values
- High influent TKN/COD ratios and associated 2 anoxic reactor high NO_{x+3}^- concentrations, result in higher DSVI values
- An increase in DSVI resulted in a decrease in VSS and a decrease in DSVI resulted in an increase in VSS
- Filament type 021N proliferated with septic sewage
- Filament type 0092 proliferated with high concentrations of NO_{x+3}^- in 2nd anoxic reactor

Table B.19

SYSTEM 16 :		CHANGES IN OPERATING CONDITIONS
Period (Days)	Operating conditions	Reasons for changes in operating conditions: Comments on system response
1 - 49	MUCT configuration 33% aerated, high TKN/COD (0,12-0,14 mgN/mgCOD) from NH_4^+ addition to influent	To develop a sludge with high DSVI containing filament type 0092: DSVI decreased from ≈ 250 to ≈ 200 ml/g; dominant filament type 0092.
50 - 77	MUCT configuration 33% aerated, high TKN/COD (0,12-0,14 mgN/mgCOD) from NH_4^+ addition to influent	The NH_4^+ addition was stopped to provide a system with conditions complementary to System 17: DSVI increased from ≈ 180 to ≈ 225 ml/g as a consequence of filament type 021N secondary
78 - 153	MUCT configuration 33% aerated, sewage addition to small unaerated DENOX reactor	To denitrify the floc-former intracellular denitrification intermediates NO and NO_2^-, to prevent inhibitions and filament proliferation: DSVI decreased from ≈ 150 to ≈ 100 ml/g, filament type 0092 was dominant. Note the significant increase in MLSS with decrease in DSVI (see Fig 6.9).
	SYSTEM 17	
1-49	MUCT configuration 33% aerated	To develop a sludge with filament type 0092: DSVI constant at ≈ 150 ml/g; filament type 0092 dominant.
50 - 77	MUCT configuration 33% aerated, sewage addition to small DENOX reactor	To denitrify the floc-former intracellular denitrification intermediate NO & NO2 to prevent inhibition and filament proliferation: DSVI decreased from ≈ 150 to ≈ 100 ml/g, filament type 0092 was dominant. Note the significant increase in VSS with decrease in DSVI (see Fig 6.10). The reason for the high N balances is not apparent.
78 - 155	MUCT configuration 33% aerated	DENOX reactor removed to determine if filaments proliferate in its absence: DSVI increased initially from ≈ 100 to ≈ 200 ml/g by Day 86 but inexplicably decreased to ≈ 115 ml/g by Day 155; dominant filament type 0092. It is interesting that Systems 16 and 17 were decreasing and increasing after the change in configuration on Day 77, but the DSVIs of the systems then began increasing and decreasing respectively; most likely a consequence of changes in sewage composition.

Summary

- Incorporation of a small (4% of total process volume) unaerated reactor between the 2nd anoxic and aerobic reactors with 10% of the daily sewage addition initially demonstrated promising results in the control of filament type 0092.

Table B.21

Day No. System 11	Filament abundance	Dominant	Secondary	Tertiary	Remarks
1	very common	0092	*M.parvicella*	0041, 1851, 1701	• Fungus spp. • Chains of coccoid cells
35	very common	0092	021N	0041, 0803, beads, *M.parvicella*, *Thiothrix* sp.	• Gliding algae
68	common-very common	0092	*M.parvicella*	0041, 021N, 1863, *H.hydrossis*	• Chains of large cocci
98	very common-abundant	0092	-	*M.parvicella*, 0041, *H.hydrossis*	• Chains of large coccoid cells
127	common-very common	0092	021N	*M.parvicella*, 0041	
185	abundant	0092	021N	*Nocardia* sp., 0041	• Motile algae

Table B.23

Day No. System 13	Filament abundance	Dominant	Secondary	Tertiary	Remarks
1	common-very common	0092	021N	0041, *M.parvicella*	• Chains of small Gram +ve cocci present
29	very common-abundant	0092	021N	0041, 1851, *H.hydrossis*	• Motile *algae* sp. common • *Aelosoma* sp., *Fungus* sp.
67	common-very common	0092	021N	0041, *H.hydrossis*	
110	very common	021N	0092	*M.parvicella*, 0041	• Extended finger-like zooglea • Spirochaetes common
141	abundant	021N	0041, 0092	*M.parvicella, H.hydrossis*	
172	abundant	0092	021N	0041, *H.hydrossis, M.parvicella*	• Gram positive cocci
198	abundant	021N	0092	*M.parvicella*, 0041	• Finger-like zooglea • Chains of large cocci • Algae

Table B.26

Day No. System 16	Filament abundance	Dominant	Secondary	Tertiary	Remarks
6	very common-abundant	0092	-	0041, 0803, *M.parvicella*, 021N	• Unicells common
44	very common-abundant	0092	021N	0041, 1851, *H.hydrossis*	
76	abundant	0092	021N	*H.hydrossis*, *M.parvicella*, 0041	
121	very common	0092	021N, *M.parvicella*	*Thiothrix* sp., 0041	• Chains of cocci

Table B.27

Day No. System 17	Filament abundance	Dominant	Secondary	Tertiary	Remarks
6	very common-abundant	0092	021N	0041, *M.parvicella*	• Chains of cocci
44	very common	0092	021N	0041, 0803, *M.parvicella*	• Algae • Chains of cocci
76	very common-abundant	0092	021N	Flexibacter, *M.parvicella*, 0041	• Spirochaetes very common
121	very common	0092	021N	*H.hydrossis*, *M.parvicella*, 0041	• Chains of cocci

Table B.30

NITROGEN BALANCE: SYSTEM 12

Q= 10 l/d
Vp= 20 l
Rs= 20 d
w = 1 l/d
fn= 0.1 mgN/mgVSS
fcv= 1.48 mgCOD/mgVSS

MNti: influent nitrogen
MNte: effluent nitrogen
MNw: nitrogen in waste sludge
MNoxe: eff nitrate + nitrite
MNo2e: effluent nitrite
MNoxd: nitrate+nitrite denit
MNo2d: nitrite denitrif
Xv: volatile suspended solids

a: aerobic-anoxic recycle ratio
s: sludge recycle ratio
r: anoxic-anaerobic recycle ratio
Noxox: 2nd anoxic nitrate + nitrite
No2ox: 2nd anoxic nitrite
Noxan: anaerobic nitrate + nitrite
No2an: anaerobic nitrite

Steady State Period	Day-Day	MNti mgN/d	MNte mgN/d	MNw mgN/d	MNoxe mgN/d	MNo2e mgN/d	MNoxd mgN/d	MNo2d mgN/d	NITROGEN Mass Balance
1	1- 40	1039.0	39.0	254.5	182.0		481.2		92
2	41- 63	919.4	35.5	251.1	82.7		269.4		69
3	64- 99	973.9	40.0	246.1	159.9		373.2		84
4	100-121	1082.4	40.8	237.6	253.1		599.7		105
5	151-170	1084.6	53.0	239.2	155.5		273.2		66
6	171-198	1382.0	45.1	232.8	291.7		336.4		66

Steady State Period	Day-Day	Xv mgVSS/l	a	s	r	Noxox mgN/l	No2ox mgN/l	Noxan mgN/l	No2an mgN/l	Noxe mgN/l
1	1- 40	2545	4	1	1	14.9		5.0		18.2
2	41- 63	2511	4	1	1	6.8		1.4		8.3
3	64- 99	2461	4	1	1	13.7		5.6		16.0
4	100-121	2376	4	1	1	25.9		11.5		25.3
5	151-170	2392	4	1	1	14.1		7.5		15.6
6	171-198	2328	4	1	1	27.7		17.7		29.2

Table B.31

NITROGEN BALANCE: SYSTEM 13

Q= 10 l/d
Vp= 20 l
Rs= 20 d
w = 1 l/d
fn= 0.1 mgN/mgVSS
fcv= 1.48 mgCOD/mgVSS

MNti: influent nitrogen
MNte: effluent nitrogen
MNw: nitrogen in waste sludge
MNoxe: eff nitrate + nitrite
MNo2e: effluent nitrite
MNoxd: nitrate+nitrite denit
MNo2d: nitrite denitrif
Xv: volatile suspended solids

a: aerobic-anoxic recycle ratio
s: sludge recycle ratio
r: anoxic-anaerobic recycle ratio
Noxox: 2nd anoxic nitrate + nitrite
No2ox: 2nd anoxic nitrite
Noxan: anaerobic nitrate + nitrite
No2an: anaerobic nitrite

Steady State Period	Day-Day	MNti mgN/d	MNte mgN/d	MNw mgN/d	MNoxe mgN/d	MNo2e mgN/d	MNoxd mgN/d	MNo2d mgN/d	NITROGEN Mass Balance
1	1- 40	1042.8	43.3	250.4	149.5		431.6		84
2	41- 63	1243.4	46.4	253.7	170.0		477.0		76
3	64- 99	1292.3	53.0	246.0	214.2		436.7		74
4	100-121	1414.2	42.0	240.7	318.7		493.7		77
5	151-170	1420.7	36.0	229.4	282.3		365.7		64
6	171-198	1074.7	46.9	241.3	177.2		458.4		86

Steady State Period	Day-Day	Xv mgVSS/l	a	s	r	Noxox mgN/l	No2ox mgN/l	Noxan mgN/l	No2an mgN/l	Noxe mgN/l
1	1- 40	2504	4	1	1	13.5		4.3		14.9
2	41- 63	2537	4	1	1	16.2		5.7		17.0
3	64- 99	2460	4	1	1	19.4		9.2		21.4
4	100-121	2407	4	1	1	33.3		19.3		31.9
5	151-170	2294	4	1	1	26.5		16.3		28.2
6	171-198	2413	4	1	1	15.5		5.6		17.7

Table B.34

NITROGEN BALANCE: SYSTEM 16

Q= 10 l/d
Vp= 20 l
Rs= 20 d
w = 1 l/d
fn= 0.1 mgN/mgVSS
fcv= 1.48 mgCOD/mgVSS

MNti: influent nitrogen
MNte: effluent nitrogen
MNw: waste sludge nitrogen
MNo3e: effluent nitrate
MNo2e: effluent nitrite
MNo3d: nitrate denitrified
MNo2d: nitrite denitrified
Xv: volatile suspended solids

a: aerobic-anoxic recycle ratio
s: sludge recycle ratio
r: anoxic-anaerobic recycle ratio
No3ox: 2nd anoxic nitrate
No2ox: 2nd anoxic nitrite
No3an: anaerobic nitrate
No2an: anaerobic nitrite

Steady State Period	Day-Day	MNti mgN/d	MNte mgN/d	Xv mgVSS/l	MNw mgN/d	MNo3e mgN/d	MNo2e mgN/d	MNo3d mgN/d	MNo2d mgN/d	NITROGEN Mass Balance
1	1- 17	1421	57	2802	280	435	1	804	-60	107
2	18- 49	1354	53	2668	267	492	1	722	-32	111
3	50- 77	1383	51	2772	277	517	1	793	-11	118
4	78- 97	941	44	2774	277	234	2	579	-1	121
5	98-124	973	46	2996	300	252	1	633	-2	126
6	125-153	911	40	2990	299	162	-	383	-	97

Steady State Period	Day-Day	a	s	r	No3ox mgN/l	No2ox mgN/l	No3an mgN/l	No2an mgN/l	No3e mgN/l	No2e mgN/l
1	1- 17	4	1	1	43.1	0.3	22.6	1.2	43.5	0.1
2	18- 49	4	1	1	51.1	0.3	30.2	0.8	49.2	0.1
3	50- 77	4	1	1	50.6	0.2	29.1	0.4	51.7	0.1
4	78- 97	2	1	1	23.7	0.1	3.2	0.1	23.4	0.2
5	98-124	2	1	1	25.3	0.2	3.1	0.2	25.2	0.1
6	125-153	2	1	1	14.0	-	1.5	-	16.2	-

Table B.35

NITROGEN BALANCE: SYSTEM 17

Q= 10 l/d
Vp= 20 l
Rs= 20 d
w = 1 l/d
fn= 0.1 mgN/mgVSS
fcv= 1.48 mgCOD/mgVSS

MNti: influent nitrogen
MNte: effluent nitrogen
MNw: waste sludge nitrogen
MNo3e: effluent nitrate
MNo2e: effluent nitrite
MNo3d: nitrate denitrified
MNo2d: nitrite denitrified
Xv: volatile suspended solids

a: aerobic-anoxic recycle ratio
s: sludge recycle ratio
r: anoxic-anaerobic recycle ratio
No3ox: 2nd anoxic nitrate
No2ox: 2nd anoxic nitrite
No3an: anaerobic nitrate
No2an: anaerobic nitrite

Steady State Period	Day-Day	MNti mgN/d	MNte mgN/d	Xv mgVSS/l	MNw mgN/d	MNo3e mgN/d	MNo2e mgN/d	MNo3d mgN/d	MNo2d mgN/d	NITROGEN Mass Balance
1	1- 17	1040	49	2913	291	205	2	565	-7	106
2	18- 49	980	37	2748	275	214	2	591	-7	113
3	50- 77	950	41	2922	292	220	2	605	-5	122
4	78- 97	939	50	2555	255	192	1	498	-1	106
5	98-124	972	47	2773	277	232	1	570	-2	116
6	125-153	918	41	2749	275	123	-	310	-	82

Steady State Period	Day-Day	a	s	r	No3ox mgN/l	No2ox mgN/l	No3an mgN/l	No2an mgN/l	No3e mgN/l	No2e mgN/l
1	1- 17	2	1	1	23.8	0.2	2.9	0.3	20.5	0.2
2	18- 49	2	1	1	23.8	0.2	2.5	0.3	21.4	0.2
3	50- 77	2	1	1	24.3	0.2	2.6	0.2	22.0	0.2
4	78- 97	2	1	1	18.7	0.2	1.7	0.2	19.2	0.1
5	98-124	2	1	1	21.2	0.3	2.2	0.2	23.2	0.1
6	125-153	2	1	1	11.8	-	1.3	-	12.3	-

(iv) Mass of nitrate and nitrite in effluent: MN_{o3e}, MN_{o2e}

$MN_{o3e} = N_{o3e} \cdot Q$

where

N_{o3e} = concentration of NO_3^- in effluent (mgN/ℓ)

$MN_{o2e} = N_{o2e} \cdot Q$

where

N_{o2e} = concentration of NO_2^- in effluent (mgN/ℓ)

(v) Mass of nitrogen in waste sludge: MN_w

$MN_w = f_n \, M\Delta X_v$

where

f_n = nitrogen fraction of VSS (mgN/mgVSS)
= 0,1 mgN/mgVSS

$M\Delta X_v$ = VSS in waste sludge (mgVSS)
$= 1/R_s \cdot V \cdot X_v$

where

V = volume of system (ℓ)

R_s = sludge age (d)

X_v = volatile suspended solids (mg/ℓ)

$$\% \text{ N balance} = 100 \, (MN_{o2d}+MN_{o3d}+MN_{te}+MN_{o2e}+MN_{o3e})/(MN_{ti}+MN)$$

$MN_{ti} = 10 \text{ ℓ/d} \cdot 137{,}5 \text{ mgN/ℓ} = 1375 \text{ mgN/d}$

$MN_{o2d} = (4 \cdot 0{,}6 + 1 \cdot 0{,}3) \cdot 10 - (1 + 1 + 4) \cdot 10 \cdot 2{,}7 = -135$

$MN_{o3d} = (4 \cdot 30{,}7 + 1 \cdot 33{,}9) \cdot 10 - (1 + 1 + 4) \cdot 10 \cdot 13{,}5 = 757$

$MN_e = (5{,}9 + 0{,}3 + 33{,}9) \text{ mgN/ℓ} \cdot 10 \text{ ℓ/d} = 401 \text{ mgN/d}$

$MN_w = 0{,}1 \cdot 1\text{ℓ} \cdot 2391 \text{ mgVSS/ℓ} = 239$

$$\% \text{ N balance} = 100 \, (-135+757+401+239)/1375 = 92$$

denitrification. In some batch tests (2, 3, 4, 11, 12, 13, 14, 15), denitrification was assisted by the addition of a small volume (0,25 ℓ) of sewage at the start of the unaerated period. In later batch tests, this procedure was discontinued and high concentrations of NO_2^- and NO_3^- in the sludge were removed by "washing" the sludge, i.e. diluting the sludge to 1 ℓ, settling for $^1/_2$ h, decanting, then repeating the procedure twice. *Thiourea* (10 mg/ℓ – final batch volume) was usually added during this unaerated period and for tests operated without an unaerated period, it was added during the aerated pre–test period.

Aerobic pre–test conditions: The sludge was exposed to an aerated period of 2 h during which time NO_2^- was added so as to induce inhibition in the sludge through aerobic denitrification of NO_2^-. The concentration of DO was maintained between 2 and 5 mgO/ℓ during this period and also during the period following substrate addition.

Origin of sludge

For anoxic–aerobic systems irrespective of the configuration, sludge was taken from an unaerated zone, i.e. during the anoxic period of an IAND system, from the anoxic reactor of a 2RND system, and from the 2nd anoxic reactor of an MUCT system. Sludge was taken from the unaerated as opposed to the aerated zone in these systems to avoid high concentrations of NO_2^- and NO_3^-.

Calculation of inhibition

The degree of inhibition induced in a sludge is determined by calculating the fraction of the maximum OUR which is inhibited immediately after feeding. For example, for Batch test A conducted on sludge from System 7 on Day 258, the OUR increases from 9,5 to 18,8 mgO/gVSS.h over a period of $5^1/_2$ h and the inhibition is calculated as follows:

$$100 \cdot (18{,}8 - 9{,}5)/18{,}8 = 49\%$$

Results

2RND configuration (System 9): Tables B.36 to B.54 indicate the operating conditions for Batch tests 1 to 19 respectively, and Figs B.14 to B.32 illustrate the results of Batch tests 1 to 19 respectively.

IAND configuration (System 5): Tables B.55 and B.56 indicate the operating

(1) *The effect of different concentrations of NO_2^- at the end of the anoxic period, just before substrate addition under aerobic conditions*: Four tests were conducted, with different concentrations of NO_2^- present at the time of substrate addition, different percent inhibitions were induced; BT 2 (25,0 mgN/ℓ, 68%), BT 4 (5,5 mgN/ℓ, 36%), BT 5 (0,5 mgN/ℓ, 30%) and BT 11 (0,1 mgN/ℓ, 8%).

- Inhibition of OUR was proportional to the concentration of NO_2^-; as the concentration of NO_2^- decreased from 25,0 to 0,1 mgN/ℓ, inhibition decreased from 68 to 8%.

(2) *Comparison of inhibition induced from denitrification of NO_3^- and NO_2^- under aerobic conditions*: Three tests can be compared to determine the extent of aerobic denitrification of NO_3^- and NO_2^-; Batch test 12 (NO_3^- added under aerobic conditions, no inhibition), Batch test 13 (NO_2^- added under aerobic conditions, 65% inhibition), and Batch test 1 (no NO_2^- or NO_3^- added under aerobic conditions, no inhibition).

- NO_3^- is not, but NO_2^- is denitrified under aerobic conditions, resulting in inhibition.

(3) *Effect of RBCOD and SBCOD on induction of inhibition through aerobic denitrification of NO_2^-*: Effect of RBCOD and SBCOD on induction of inhibition through aerobic denitrification of NO_2^-. Two tests can be compared to determine the effect of RBCOD and SBCOD on aerobic denitrification of NO_2^-; Batch test 13 (NO_2^- added under aerobic conditions with SBCOD only present, 65% inhibition) and Batch test 17 (NO_2^- added under aerobic conditions with RBCOD only present, 6% inhibition).

- Inhibition is induced with NO_2^- and SBCOD, but is not induced with NO_2^- and RBCOD present under aerobic conditions. The relief of inhibition with time in Batch test 13 after feeding indicates that RBCOD not only prevents inhibition but also relieves it.

(4) *Time required for aerobic denitrification of NO_2^- for induction of inhibition*: Three tests can be compared to determine the effect of time for NO_2^- denitrification under aerobic conditions; Batch test 14 (NO_2^- present for 30

inhibition initially increased rapidly (1–4 days) to ≈ 80%, then decreased (2–8 days) to ≈ 40% before increasing (15–30 days) to ≈ 70%. In changing from anoxic to aerobic conditions, the level of inhibition decreased (40 days) from ≈ 70% to ≈ 20%.

- Changes between anoxic and aerobic conditions result in changes in synthesis of anoxic and aerobic respiratory enzymes. These results are discussed at length in Chapter 5.

(7) *Inhibition of sludge from IAND and MUCT systems*: Two tests conducted on sludges from an IAND system, one with NO_2^- present under anoxic pre–test conditions (Batch test 20, 51% inhibited), the other with NO_2^- present under anoxic pre–test conditions (Batch test 21, no inhibition), demonstrated similar results to sludges from 2RND systems. Two tests conducted on sludges from the 2nd anoxic reactor of an MUCT system, one with NO_2^- present under aerobic pre–conditions (Batch test 25, 29% inhibition), the other without NO_2^- present in the sludge (Batch test 22, no inhibition) demonstrated similar results to sludges from IAND and 2RND systems.

- Inhibition can be induced in sludge from ND and NDBEPR systems irrespective of the configuration.

(8) *Inhibition induced in sludge from either aerobic or anoxic reactors*: Two tests conducted on sludges from the aerobic reactor of an MUCT system, one with NO_2^- present under aerobic pre–conditions (Batch test 24, 18% inhibition), the other without NO_2^- present in the sludge (Batch test 23, no inhibition) demonstrated similar results to sludges from the aerobic reactor of MUCT systems (Batch tests 25 and 22).

- Inhibition can be induced in sludges from either the aerobic or anoxic reactors of an NDBEPR system.

Aspects (1) to (8) above have considerable implications with regard to the biochemical respiratory model for facultative organisms. Aspects (9) to (12) below describe experimental factors which although not directly implicated in the biochemical respiratory model, were examined to determine their effect on the results of aerobic inhibition batch tests.

NO_2^- (*Nitrosomonas*) and not the second nitrification step, $NO_2^- \rightarrow NO_3^-$ *(Nitrobacter)*.

Conceivably the increase in OUR after substrate addition could result from an increase in *Nitrobacter*. However it should be noted that in Batch test 17 in which NO_2^- was added (after 5 h) when the maximum OUR had been reached, the OUR increased immediately by 3,6 mgO/gVSS, an increase attributable to nitrification of NO_2^-. Examination of the oxygen requirements for nitrification of $NO_2^- \rightarrow NO_3^-$ (1,14 mgO/mgNO_2^-) calculated from the change in NO_3^- concentration after the addition of NO_2^- for Batch tests 13, 9, 6, 4, and 2, yielded oxygen consumption values of 3,0; 1,0; 1,3; 4,7; and 6,1 mgO/gVSS.h respectively. These values are insignificant compared to many of the increases in OUR.

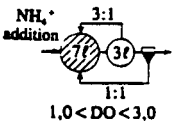
- Increases > 3,5 mgO/gVSS.h in OUR in batch tests are not a consequence of nitrification of NO_2^-.

Anoxic batch tests

Procedures and results

Anoxic, denitrification batch tests, conducted to determine the extent of synthesis of denitrification enzymes, follow the procedures described by Ekama *et al.* (1986) for determination of nitrate reduction rates. These tests were conducted on System 7, during periods when the system was continuous anoxic and continuous aerobic. The conditions of operation of Batch tests DBT1 to DBT4 are shown in Tables B.79 to B.82, and the results of the batch tests are illustrated in Figs B.57 to B.60.

Table B.37

BATCH TEST 2 OPERATING CONDITIONS		
Objective: To determine OUR response following NO_2^- addition under anoxic pre-test conditions to give NO_2^- (25,0 mgN/ℓ) at start of aerobic conditions.		
PARENT SYSTEM CONDITIONS		
System 9; Day 227 **System configuration: 2RND (MLE)**		
Sludge age	**(d)**	**15**
BATCH TEST CONDITIONS		
Sludge volume	**(ℓ)**	**1;0**
Water volume	**(ℓ)**	**1,0**
Sewage volume	**(ℓ)**	**0,4 @ t=0; 0,8 @ t=2**
Total volume	**(ℓ)**	**3,0**
MLSS	**(mg/ℓ)**	**626**
VSS	**(mg/ℓ)**	**510**
F/M	**(mgCOD/mgVSS)**	**0,6**
Anoxic pre-test period	**(h)**	**2,0**
Aerobic pre-test period	**(h)**	**-**
DO	**(mgO/ℓ)**	**2 - 5**
Temperature	**(°C)**	**20**
SEWAGE TO BATCH TEST		
COD	**(mgCOD/ℓ-final batch vol)**	**307**
TKN	**(mgN/ℓ-final batch volume)**	**28,3**

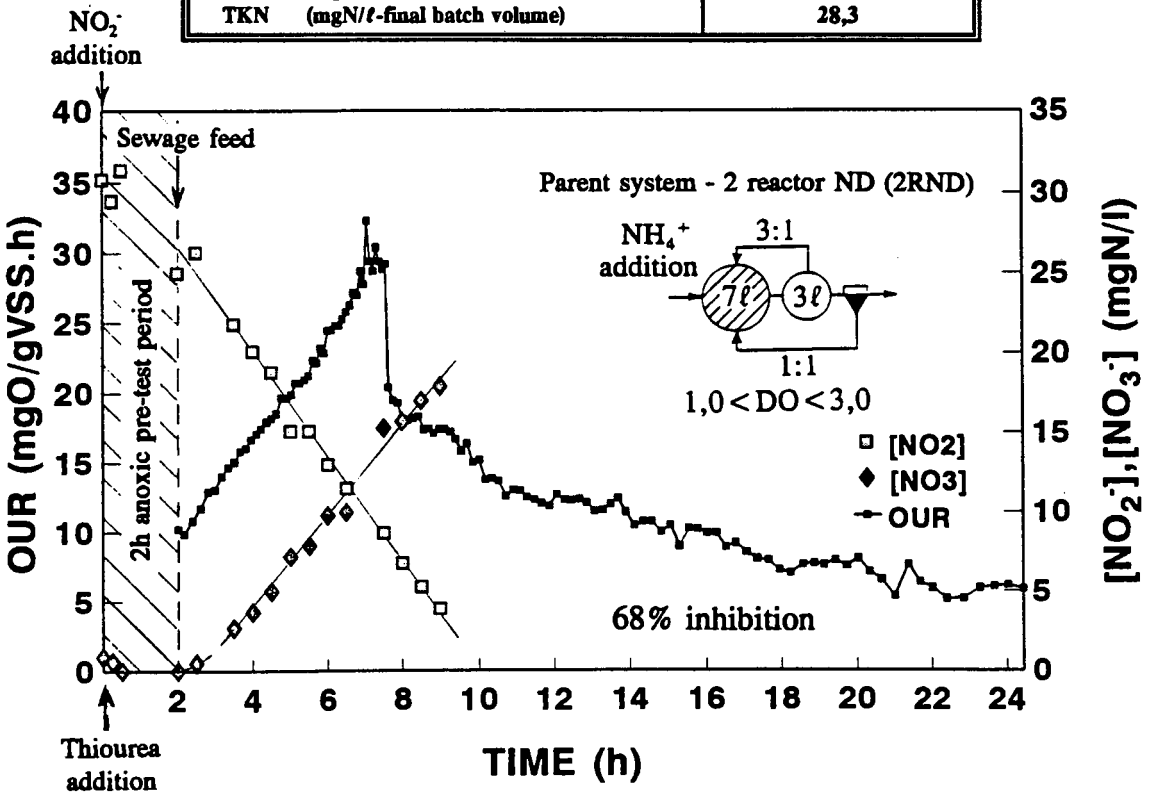
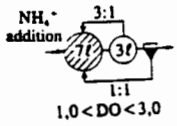


Fig B.15

Table B.39

BATCH TEST 4 OPERATING CONDITIONS		
Objective: To determine OUR response following NO_2^- addition under anoxic pre-test conditions to give NO_2^- (5,5 mgN/ℓ) at start of aerobic conditions.		
PARENT SYSTEM CONDITIONS		
System 9; Day 233 System configuration: 2RND (MLE)		
Sludge age	(d)	15
BATCH TEST CONDITIONS		
Sludge volume	(ℓ)	0,8
Water volume	(ℓ)	1,2
Sewage volume	(ℓ)	0,25 @ t=0;0,75 @ t=2
Total volume	(ℓ)	3,0
MLSS	(mg/ℓ)	490
VSS	(mg/ℓ)	451
F/M	(mgCOD/mgVSS)	0,6
Anoxic pre-test period	(h)	2,0
Aerobic pre-test period	(h)	-
DO	(mgO/ℓ)	2 - 5
Temperature	(°C)	20
SEWAGE TO BATCH TEST		
COD	(mgCOD/ℓ-final batch vol)	288
TKN	(mgN/ℓ-final batch volume)	21,3

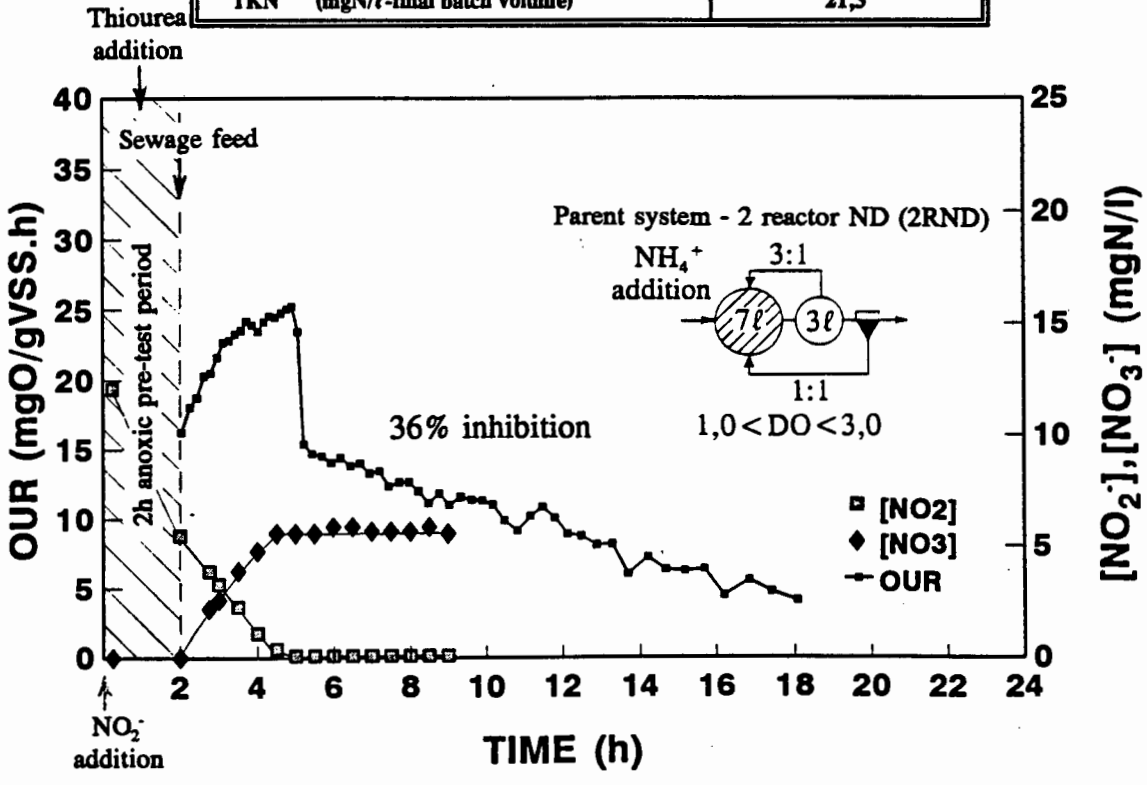
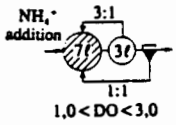


Fig B.17

Table B.41

BATCH TEST 6 OPERATING CONDITIONS		
Objective: To determine the effect of doubling the concentration of thiourea on OUR response following NO_2^- addition under aerobic pre-test period.		
PARENT SYSTEM CONDITIONS		
System 9; Day 241 System configuration: 2RND (MLE)		
Sludge age	(d)	15
BATCH TEST CONDITIONS		
Sludge volume	(ℓ)	0,8
Water volume	(ℓ)	1,2
Sewage volume	(ℓ)	1,0
Total volume	(ℓ)	3,0
MLSS	(mg/ℓ)	631
VSS	(mg/ℓ)	567
F/M	(mgCOD/mgVSS)	0,7
Anoxic pre-test period	(h)	-
Aerobic pre-test period	(h)	1,0
DO	(mgO/ℓ)	2 - 5
Temperature	(°C)	20
SEWAGE TO BATCH TEST		
COD	(mgCOD/ℓ-final batch vol)	376
TKN	(mgN/ℓ-final batch volume)	31,0

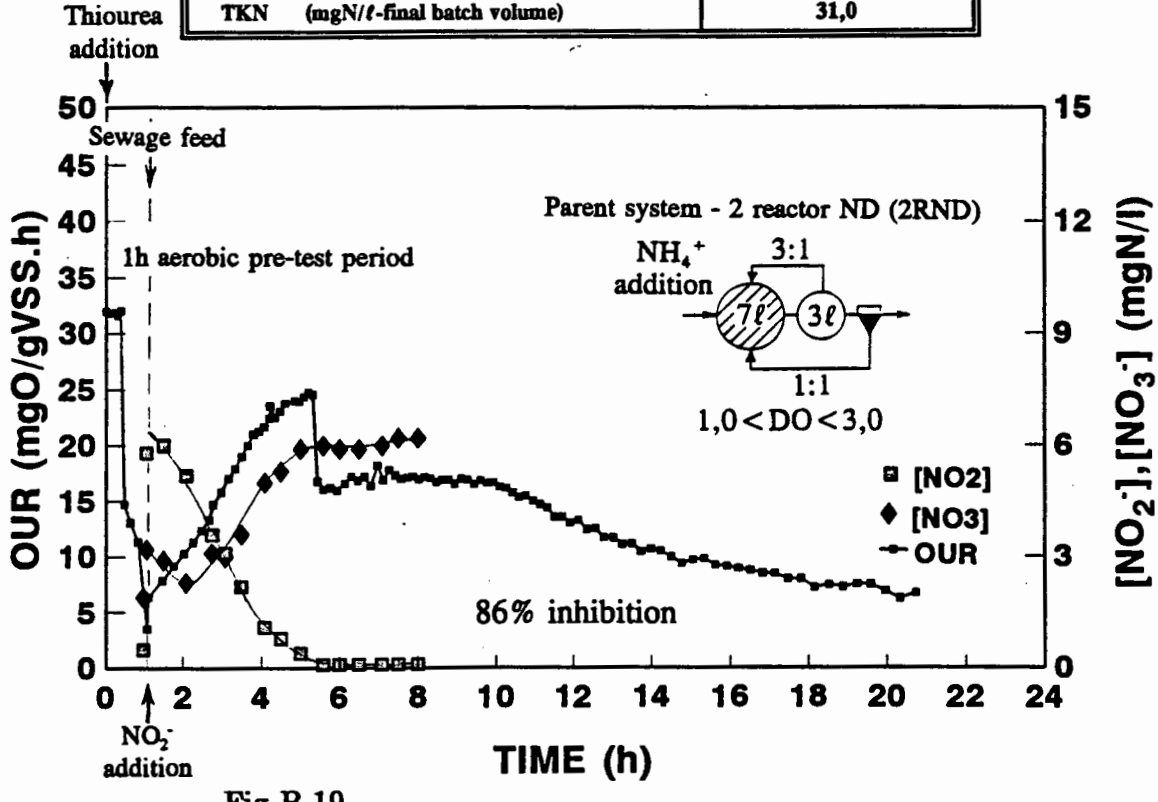
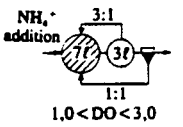


Fig B.19

Table B.43

BATCH TEST 8 OPERATING CONDITIONS		
Objective: To determine if inhibition can be re-induced in a sludge in which inhibition has been relieved.		
PARENT SYSTEM CONDITIONS		
System 9; Day 253 **System configuration: 2RND (MLE)**		
Sludge age	**(d)**	**15**
BATCH TEST CONDITIONS		
Sludge volume	**(ℓ)**	**From Batch Test 13**
Water volume	**(ℓ)**	**1,0**
Sewage volume	**(ℓ)**	**1,0**
Total volume	**(ℓ)**	**3,0**
MLSS	**(mg/ℓ)**	**459**
VSS	**(mg/ℓ)**	**409**
F/M	**(mgCOD/mgVSS)**	**0,3**
Anoxic pre-test period	**(h)**	**See end of Batch Test 20**
Aerobic pre-test period	**(h)**	**See end of Batch Test 20**
DO	**(mgO/ℓ)**	**2 - 5**
Temperature	**(°C)**	**20**
SEWAGE TO BATCH TEST		
COD	**(mgCOD/ℓ-final batch vol)**	**392**
TKN	**(mgN/ℓ-final batch volume)**	**33,4**

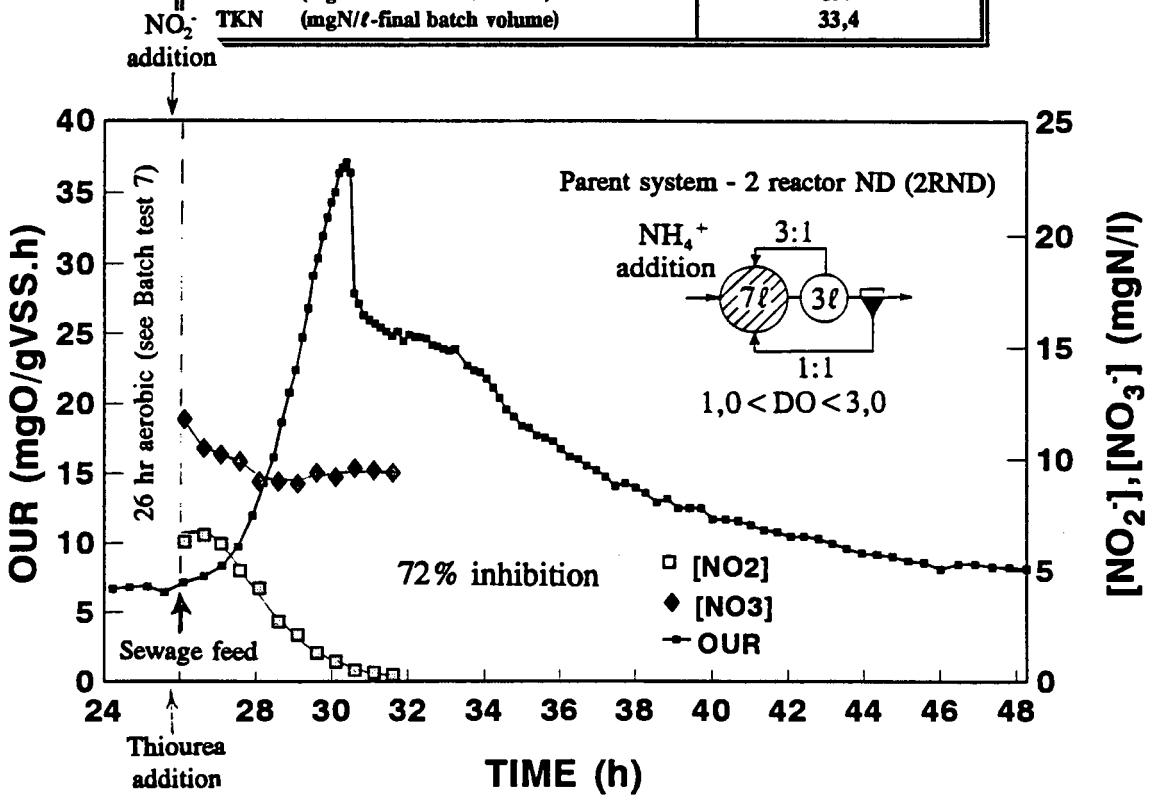
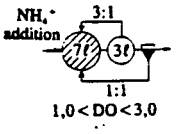


Fig B.21

Table B.45

BATCH TEST 10 OPERATING CONDITIONS		
Objective: To determine OUR response to absence of NO_2^-/NO_3^- in aerobic pre-test period.		
PARENT SYSTEM CONDITIONS		
System 9; Day 262 System configuration: 2RND (MLE)		
Sludge age	(d)	15
BATCH TEST CONDITIONS		
Sludge volume	(ℓ)	0,8
Water volume	(ℓ)	1,3
Sewage volume	(ℓ)	0,9
Total volume	(ℓ)	3,0
MLSS	(mg/ℓ)	595
VSS	(mg/ℓ)	511
F/M	(mgCOD/mgVSS)	0,7
Anoxic pre-test period	(h)	12,0
Aerobic pre-test period	(h)	2,0
DO	(mgO/ℓ)	2 - 5
Temperature	(°C)	20
SEWAGE TO BATCH TEST		
COD	(mgCOD/ℓ-final batch vol)	353
TKN	(mgN/ℓ-final batch volume)	30,1

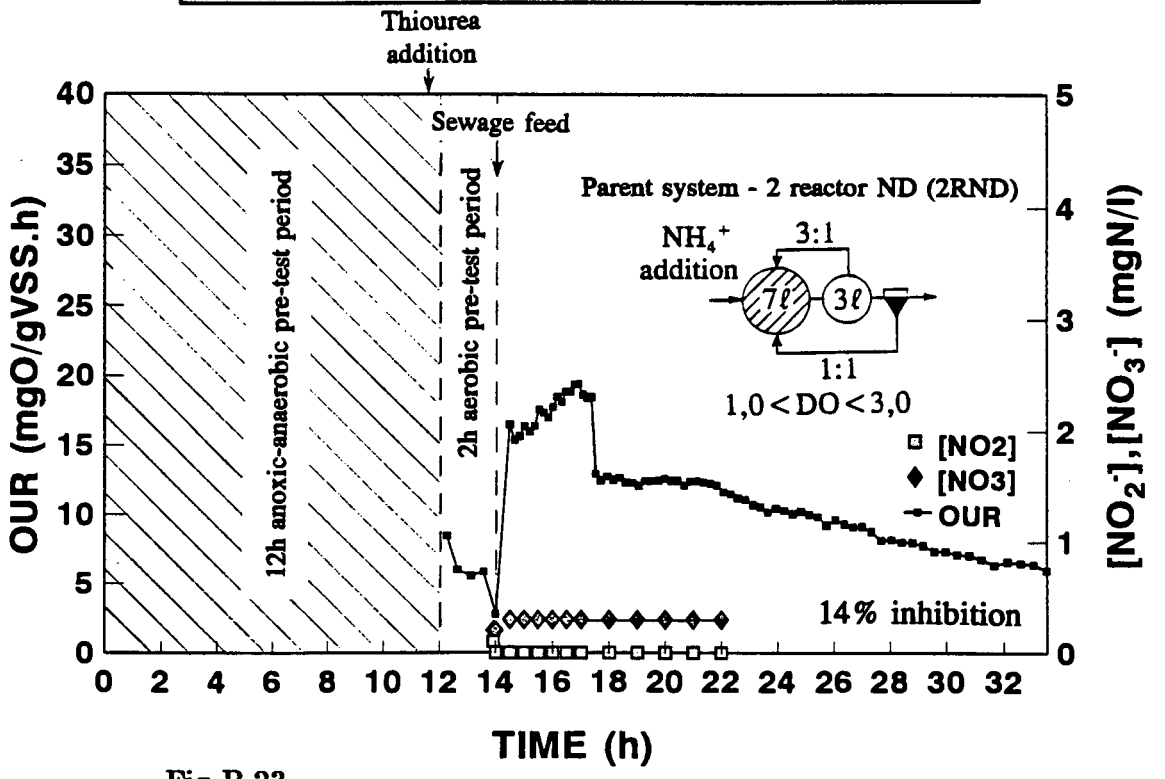
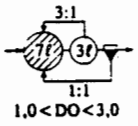


Fig B.23

Table B.47

BATCH TEST 12 OPERATING CONDITIONS		
Objective: To determine OUR response following NO_3^- (≈ 20 mgN/ℓ) addition under aerobic pre-test conditions 60 minutes before substrate addition.		
PARENT SYSTEM CONDITIONS		
System 9; Day 284 System configuration: 2RND (MLE)		
Sludge age	(d)	15
BATCH TEST CONDITIONS		
Sludge volume	(ℓ)	1,0
Water volume	(ℓ)	1,0
Sewage volume	(ℓ)	0,25 @ t=0;0,75 @ t=4
Total volume	(ℓ)	3,0
MLSS	(mg/ℓ)	641
VSS	(mg/ℓ)	594
F/M	(mgCOD/mgVSS)	0,5
Anoxic pre-test period	(h)	2,0
Aerobic pre-test period	(h)	2,0
DO	(mgO/ℓ)	2 - 5
Temperature	(°C)	20
SEWAGE TO BATCH TEST		
COD	(mgCOD/ℓ-final batch vol)	272
TKN	(mgN/ℓ-final batch volume)	22,5

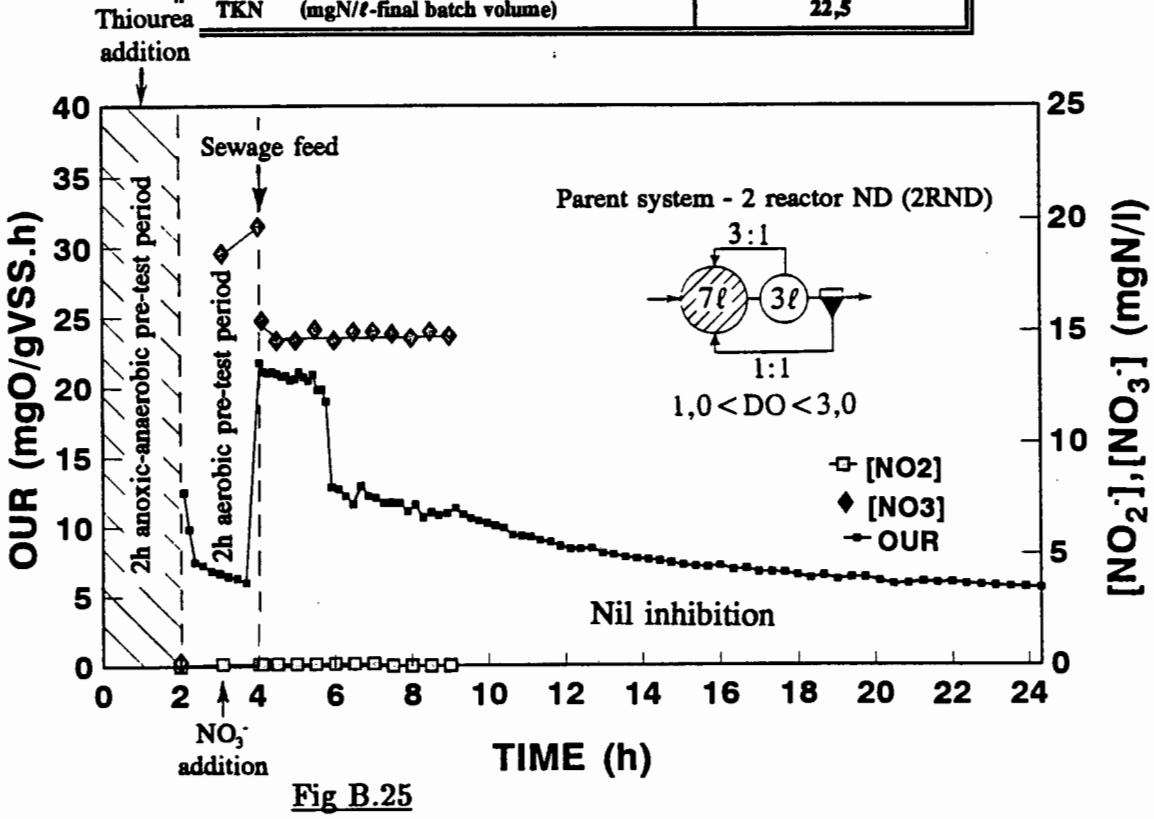
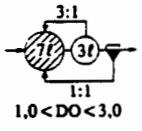


Fig B.25

Table B.49

BATCH TEST 14 OPERATING CONDITIONS		
Objective: To determine OUR response following NO_2^- (> 10 mgN/ℓ) addition under aerobic pre-test conditions 30 minutes before substrate addition.		
PARENT SYSTEM CONDITIONS		
System 9; Day 330 System configuration: 2RND (MLE)		
Sludge age	(d)	15
BATCH TEST CONDITIONS		
Sludge volume	(ℓ)	1,0
Water volume	(ℓ)	1,0
Sewage volume	(ℓ)	0,25 @ t=0;0,75 @ t=4
Total volume	(ℓ)	3,0
MLSS	(mg/ℓ)	754
VSS	(mg/ℓ)	648
F/M	(mgCOD/mgVSS)	0,5
Anoxic pre-test period	(h)	2,0
Aerobic pre-test period	(h)	2,0
DO	(mgO/ℓ)	2 - 5
Temperature	(°C)	20
SEWAGE TO BATCH TEST		
COD	(mgCOD/ℓ-final batch vol)	303
TKN	(mgN/ℓ-final batch volume)	30,0

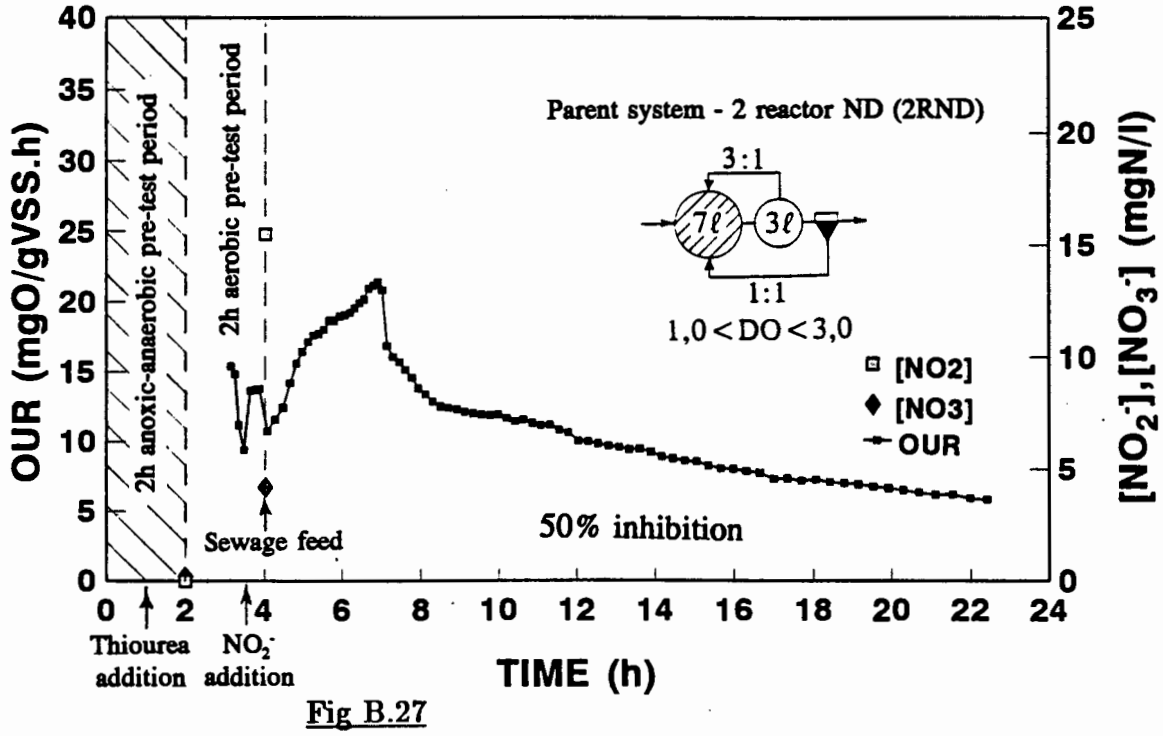
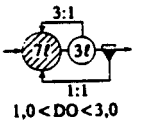


Fig B.27

Table B.51

BATCH TEST 16 OPERATING CONDITIONS		
Objective: To determine OUR response following NO_2^- addition (>10 mgN/ℓ) under aerobic pre-test conditions 5 minutes before substrate addition.		
PARENT SYSTEM CONDITIONS		
System 9; Day 334 System configuration: 2RND (MLE)		
Sludge age	(d)	15
BATCH TEST CONDITIONS		
Sludge volume	(ℓ)	1,0
Water volume	(ℓ)	1,0
Sewage volume	(ℓ)	1,0
Total volume	(ℓ)	3,0
MLSS	(mg/ℓ)	606
VSS	(mg/ℓ)	546
F/M	(mgCOD/mgVSS)	0,7
Anoxic pre-test period	(h)	2,0
Aerobic pre-test period	(h)	2,0
DO	(mgO/ℓ)	2 - 5
Temperature	(°C)	20
SEWAGE TO BATCH TEST		
COD	(mgCOD/ℓ-final batch vol)	403
TKN	(mgN/ℓ-final batch volume)	40,0

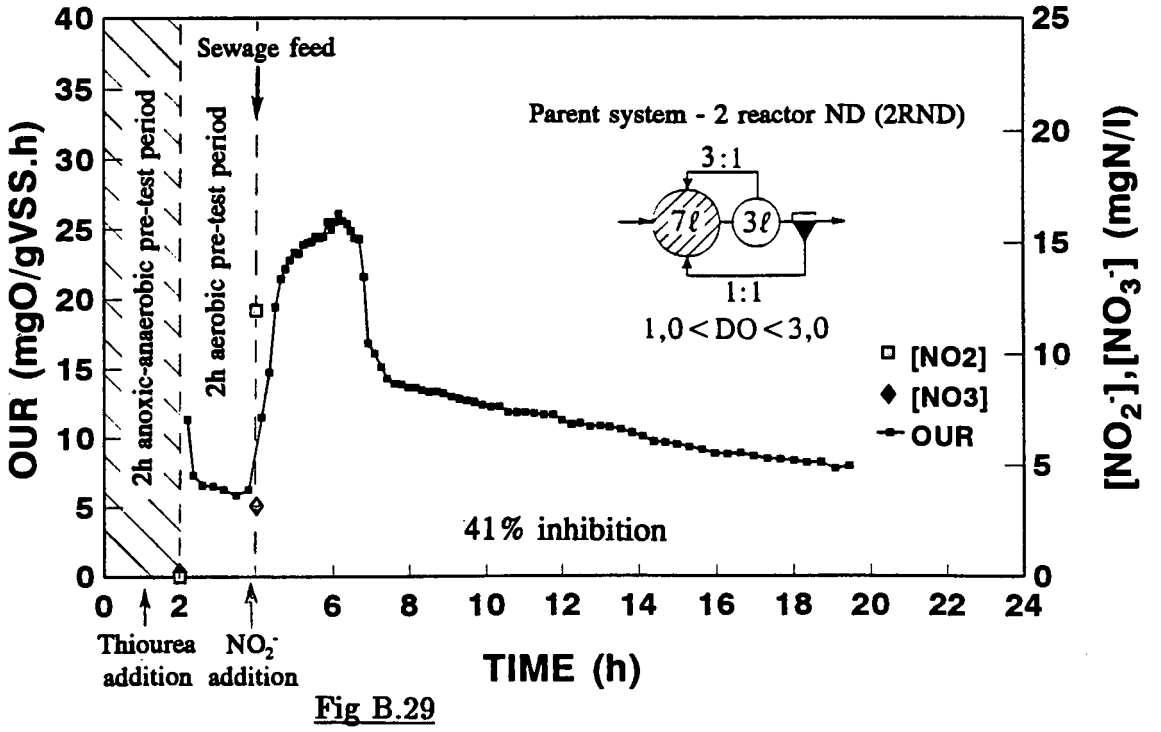
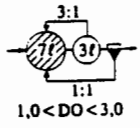


Fig B.29

Table B.53

BATCH TEST 18 OPERATING CONDITIONS		
Objective: To determine OUR response following transfer of sludge directly from parent system, i.e. no anoxic or aerobic pre-test conditions.		
PARENT SYSTEM CONDITIONS		
System 9; Day 343 **System configuration: 2RND (MLE)** **Sludge age**	**(d)**	
BATCH TEST CONDITIONS		
Sludge volume	**(ℓ)**	**1,0**
Water volume	**(ℓ)**	**1,2**
Sewage volume	**(ℓ)**	**0,8**
Total volume	**(ℓ)**	**3,0**
MLSS	**(mg/ℓ)**	**705**
VSS	**(mg/ℓ)**	**627**
F/M	**(mgCOD/mgVSS)**	**0,5**
Anoxic pre-test period	**(h)**	**-**
Aerobic pre-test period	**(h)**	**-**
DO	**(mgO/ℓ)**	**2 - 5**
Temperature	**(°C)**	**20**
SEWAGE TO BATCH TEST		
COD	**(mgCOD/ℓ-final batch vol)**	**339**
TKN	**(mgN/ℓ-final batch volume)**	**27,0**

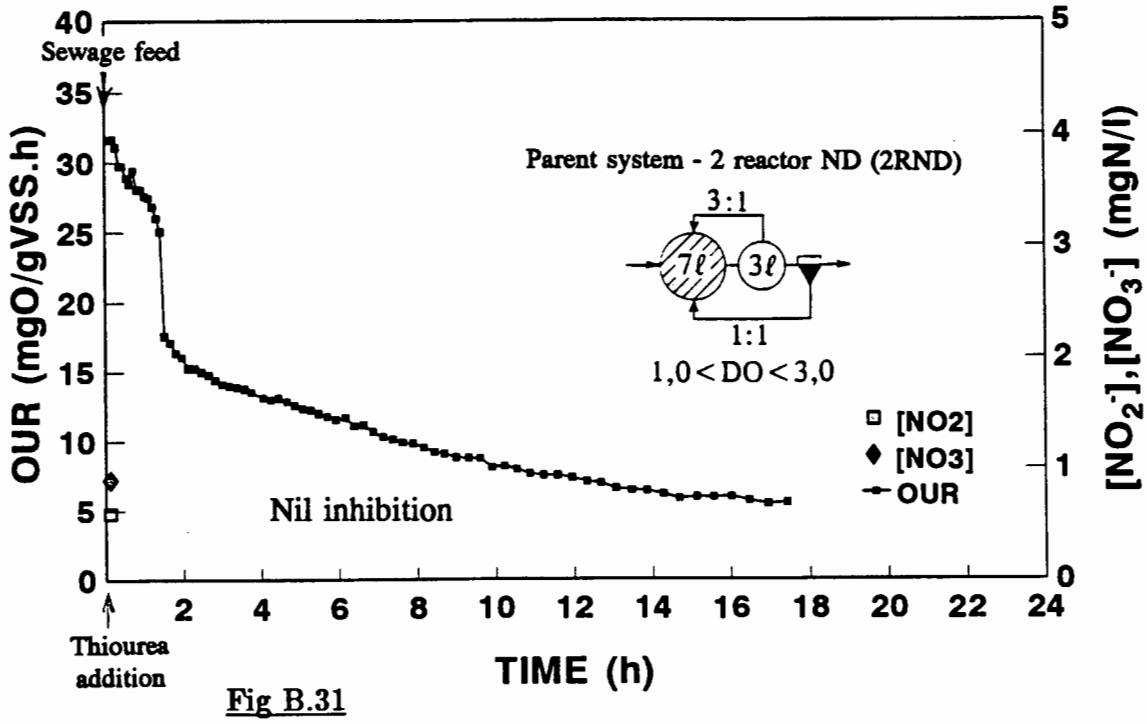


Fig B.31

Table B.55

BATCH TEST 20 OPERATING CONDITIONS		
Objective: To determine OUR response in sludge from IAND configuation following anoxic pre-test period with NO_2^- present.		
PARENT SYSTEM CONDITIONS		
System 5; Day 300 System configuration	IAND	
Sludge age	(d)	15
BATCH TEST CONDITIONS		
Sludge volume	(ℓ)	1,0
Water volume	(ℓ)	1,0
Sewage volume	(ℓ)	1,0
Total volume	(ℓ)	3,0
MLSS	(mg/ℓ)	503
VSS	(mg/ℓ)	435
F/M	(mgCOD/mgVSS)	1,0
Anoxic pre-test period	(h)	2,0
Aerobic pre-test period	(h)	-
DO	(mgO/ℓ)	2 - 5
Temperature	(°C)	20
SEWAGE TO BATCH TEST		
COD	(mgCOD/ℓ-final batch vol)	729
TKN	(mgN/ℓ-final batch volume)	41,1

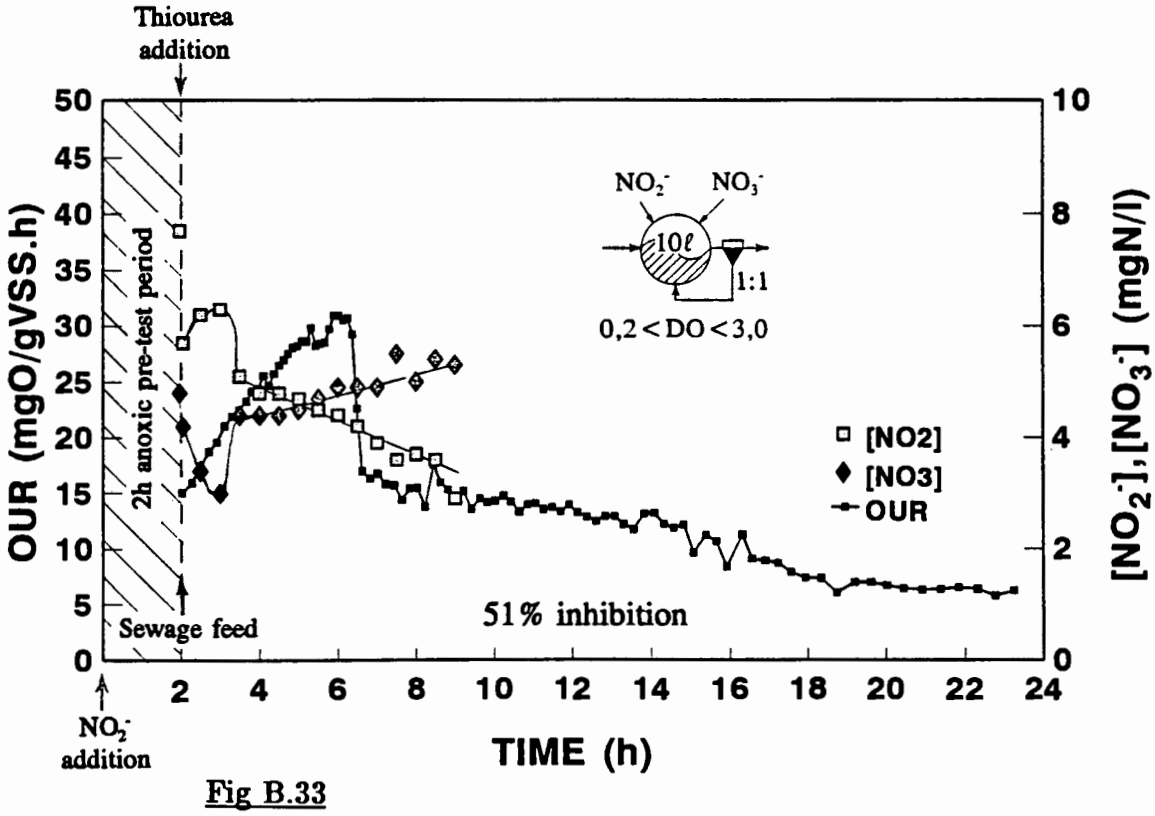
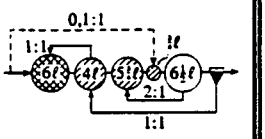


Fig B.33

Table B.57

BATCH TEST 22 OPERATING CONDITIONS		
Objective: To determine OUR response following direct transfer of sludge from 2nd anoxic reactor of parent system to batch test; i.e. anoxic or aerobic pre-test conditions.		
PARENT SYSTEM CONDITIONS		
System 17; Day 73 **System configuration: NDBEPR (MUCT)**		
Sludge age	**(d)**	**20**
BATCH TEST CONDITIONS		
Sludge volume	**(ℓ)**	**0,6**
Water volume	**(ℓ)**	**1,4**
Sewage volume	**(ℓ)**	**1,0**
Total volume	**(ℓ)**	**3,0**
MLSS	**(mg/ℓ)**	**740**
VSS	**(mg/ℓ)**	**627**
F/M	**(mgCOD/mgVSS)**	**0,7**
Anoxic pre-test period	**(h)**	**-**
Aerobic pre-test period	**(h)**	**-**
DO	**(mgO/ℓ)**	**2,0**
Temperature	**(°C)**	**2 - 5** **20**
SEWAGE TO BATCH TEST		
COD	**(mgCOD/ℓ-final batch vol)**	**423**
TKN	**(mgN/ℓ-final batch volume)**	**33,8**

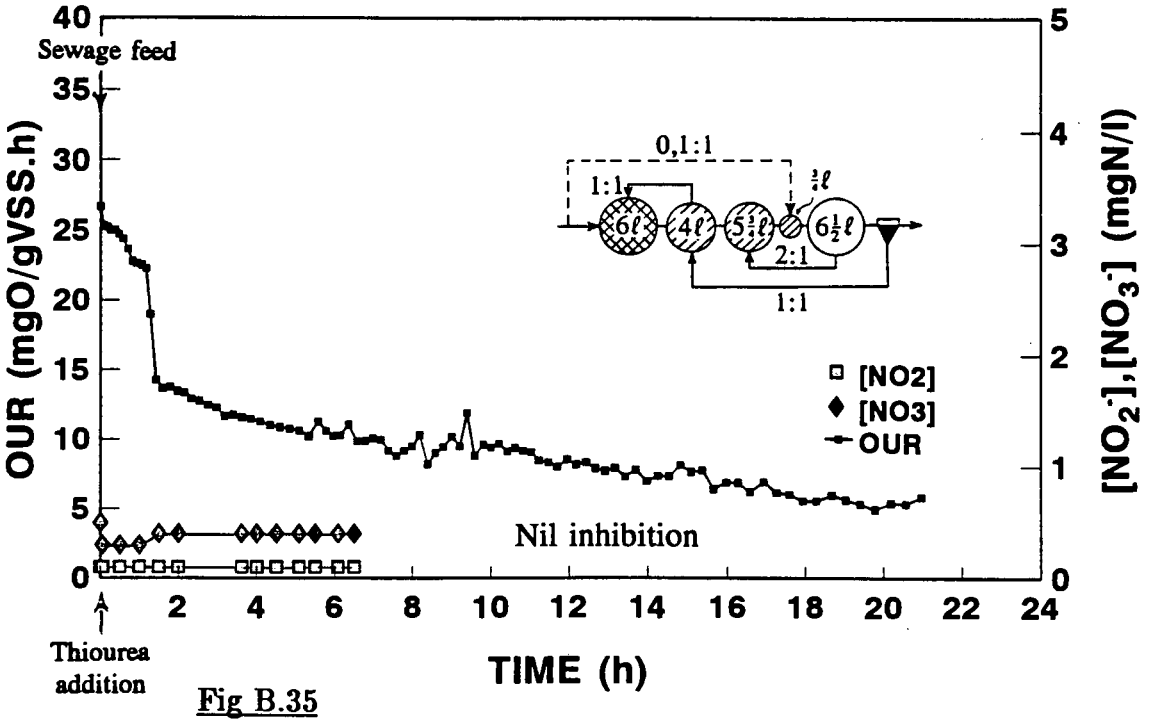
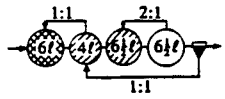


Fig B.35

Table B.59

BATCH TEST 24 OPERATING CONDITIONS		
Objective: To determine OUR response on sludge from the aerobic reactor following aerobic pre-test conditions with NO_2^- present.		
PARENT SYSTEM CONDITIONS		
System 17; Day 84 **System configuration: NDBEPR (MUCT)**		
Sludge age	**(d)**	**20**
BATCH TEST CONDITIONS		
Sludge volume	**(ℓ)**	**0,5**
Water volume	**(ℓ)**	**1,5**
Sewage volume	**(ℓ)**	**1,0**
Total volume	**(ℓ)**	**3,0**
MLSS	**(mg/ℓ)**	**707**
VSS	**(mg/ℓ)**	**655**
F/M	**(mgCOD/mgVSS)**	**0,6**
Anoxic pre-test period	**(h)**	**-**
Aerobic pre-test period	**(h)**	**2,0**
DO	**(mgO/ℓ)**	**2 - 5**
Temperature	**(°C)**	**20**
SEWAGE TO BATCH TEST		
COD	**(mgCOD/ℓ-final batch vol)**	**423**
TKN	**(mgN/ℓ-final batch volume)**	**33,8**

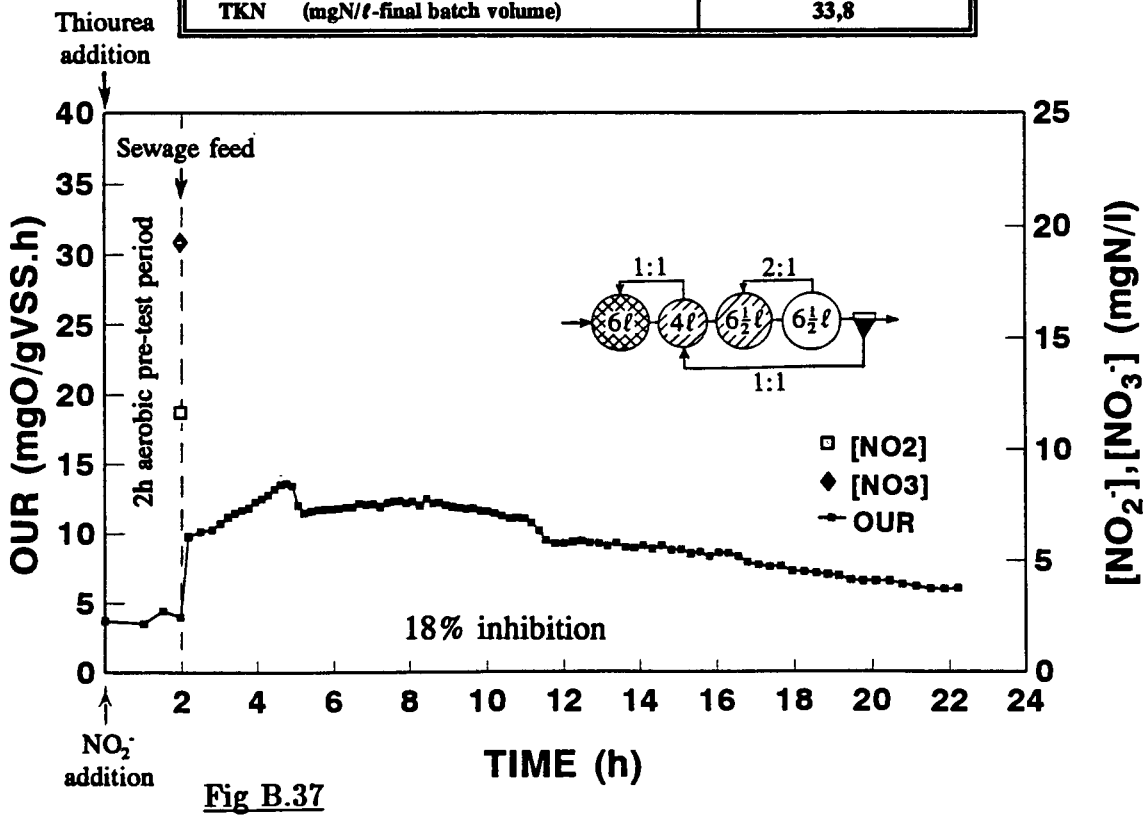
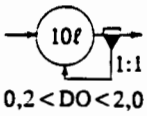


Fig B.37

Table B.61

BATCH TEST A OPERATING CONDITIONS		
Objective: To examine the effect of *anoxic* conditions on synthesis of denitrifying enzymes through analysis of inhibition of OUR due to accumulated nitric oxide (NO).		
PARENT SYSTEM CONDITIONS		
System 7; Day 258 **System configuration: continuous aerobic**		 0,2 < DO < 2,0
Sludge age	**(d)**	**15**
BATCH TEST CONDITIONS		
Sludge volume	**(ℓ)**	**0,8**
Water volume	**(ℓ)**	**1,4**
Sewage volume	**(ℓ)**	**0,8**
Total volume	**(ℓ)**	**3,0**
MLSS	**(mg/ℓ)**	**632**
VSS	**(mg/ℓ)**	**576**
F/M	**(mgCOD/mgVSS)**	**0,7**
Anoxic pre-test period	**(h)**	**-**
Aerobic pre-test period	**(h)**	**2,0**
DO	**(mgO/ℓ)**	**2 - 5**
Temperature	**(°C)**	**20**
SEWAGE TO BATCH TEST		
COD	**(mgCOD/ℓ-final batch vol)**	**385**
TKN	**(mgN/ℓ-final batch volume)**	**39,3**

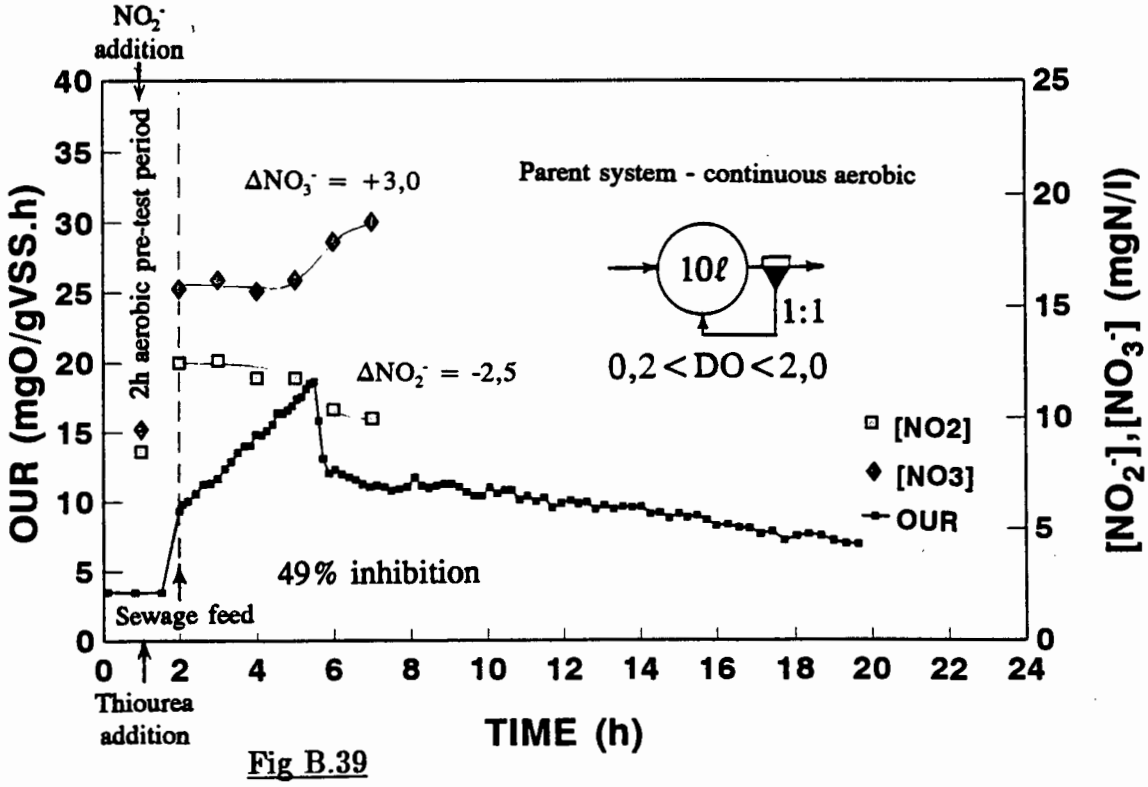
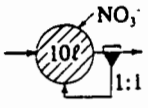


Fig B.39

Table B.63

BATCH TEST C OPERATING CONDITIONS		
Objective: To examine the effect of *anoxic* conditions on synthesis of denitrifying enzymes through analysis of inhibition of OUR due to accumulated nitric oxide (NO).		
PARENT SYSTEM CONDITIONS		
System 7; Day 269 **System configuration: Continuous anoxic**		
Sludge age	**(d)**	**15**
BATCH TEST CONDITIONS		
Sludge volume	**(ℓ)**	**0,8**
Water volume	**(ℓ)**	**1,4**
Sewage volume	**(ℓ)**	**0,8**
Total volume	**(ℓ)**	**3,0**
MLSS	**(mg/ℓ)**	**426**
VSS	**(mg/ℓ)**	**369**
F/M	**(mgCOD/mgVSS)**	**0,8**
Anoxic pre-test period	**(h)**	**1,0**
Aerobic pre-test period	**(h)**	**-**
DO	**(mgO/ℓ)**	**2 - 5**
Temperature	**(°C)**	**20**
SEWAGE TO BATCH TEST		
COD	**(mgCOD/ℓ-final batch vol)**	**301**
TKN	**(mgN/ℓ-final batch volume)**	**27,9**

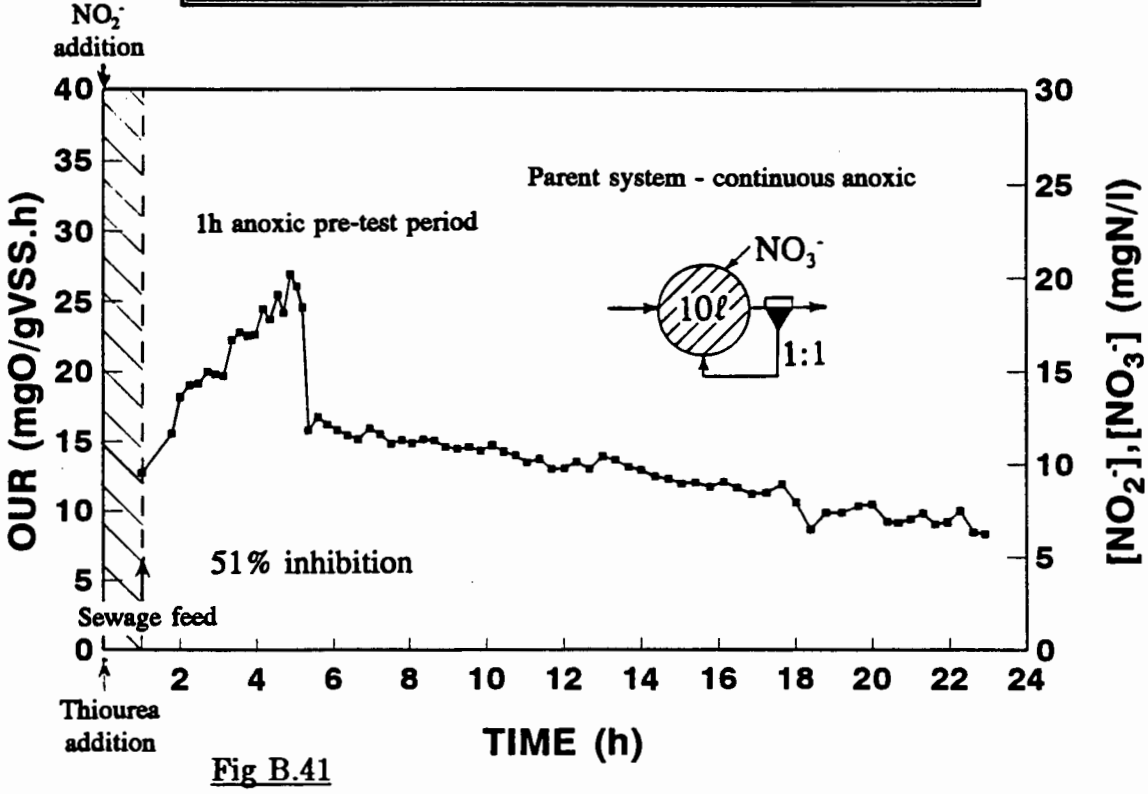
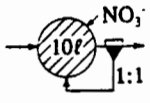


Fig B.41

Table B.65

BATCH TEST E OPERATING CONDITIONS		
Objective: To examine the effect of *anoxic* conditions on synthesis of denitrifying enzymes through analysis of inhibition of OUR due to accumulated nitric oxide (NO).		
PARENT SYSTEM CONDITIONS		
System 7; Day 284 System configuration: Continuous anoxic		
Sludge age	(d)	15
BATCH TEST CONDITIONS		
Sludge volume	(ℓ)	1,0
Water volume	(ℓ)	1,1
Sewage volume	(ℓ)	0,9
Total volume	(ℓ)	3,0
MLSS	(mg/ℓ)	565
VSS	(mg/ℓ)	444
F/M	(mgCOD/mgVSS)	0,8
Anoxic pre-test period	(h)	1,0
Aerobic pre-test period	(h)	-
DO	(mgO/ℓ)	2 - 5
Temperature	(°C)	20
SEWAGE TO BATCH TEST		
COD	(mgCOD/ℓ-final batch vol)	353
TKN	(mgN/ℓ-final batch volume)	30,1

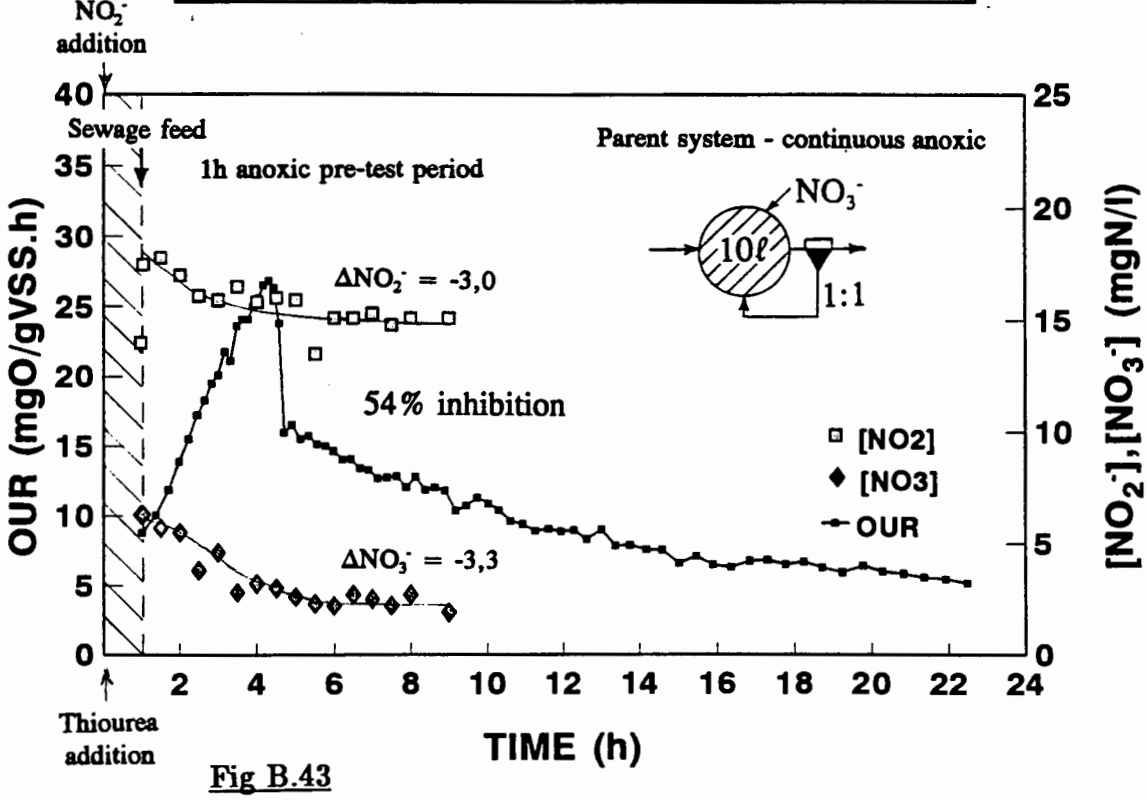
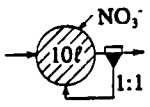


Fig B.43

Table B.67

BATCH TEST G OPERATING CONDITIONS		
Objective: To examine the effect of *anoxic* conditions on synthesis of denitrifying enzymes through analysis of inhibition of OUR due to accumulated nitric oxide (NO).		
PARENT SYSTEM CONDITIONS		
System 7; Day 304 System configuration: Continuous anoxic		
Sludge age	(d)	15
BATCH TEST CONDITIONS		
Sludge volume	(ℓ)	1,0
Water volume	(ℓ)	1,0
Sewage volume	(ℓ)	1,0
Total volume	(ℓ)	3,0
MLSS	(mg/ℓ)	366
VSS	(mg/ℓ)	349
F/M	(mgCOD/mgVSS)	1,2
Anoxic pre-test period	(h)	1,0
Aerobic pre-test period	(h)	-
DO	(mgO/ℓ)	2 - 5
Temperature	(°C)	20
SEWAGE TO BATCH TEST		
COD	(mgCOD/ℓ-final batch vol)	409
TKN	(mgN/ℓ-final batch volume)	39,1

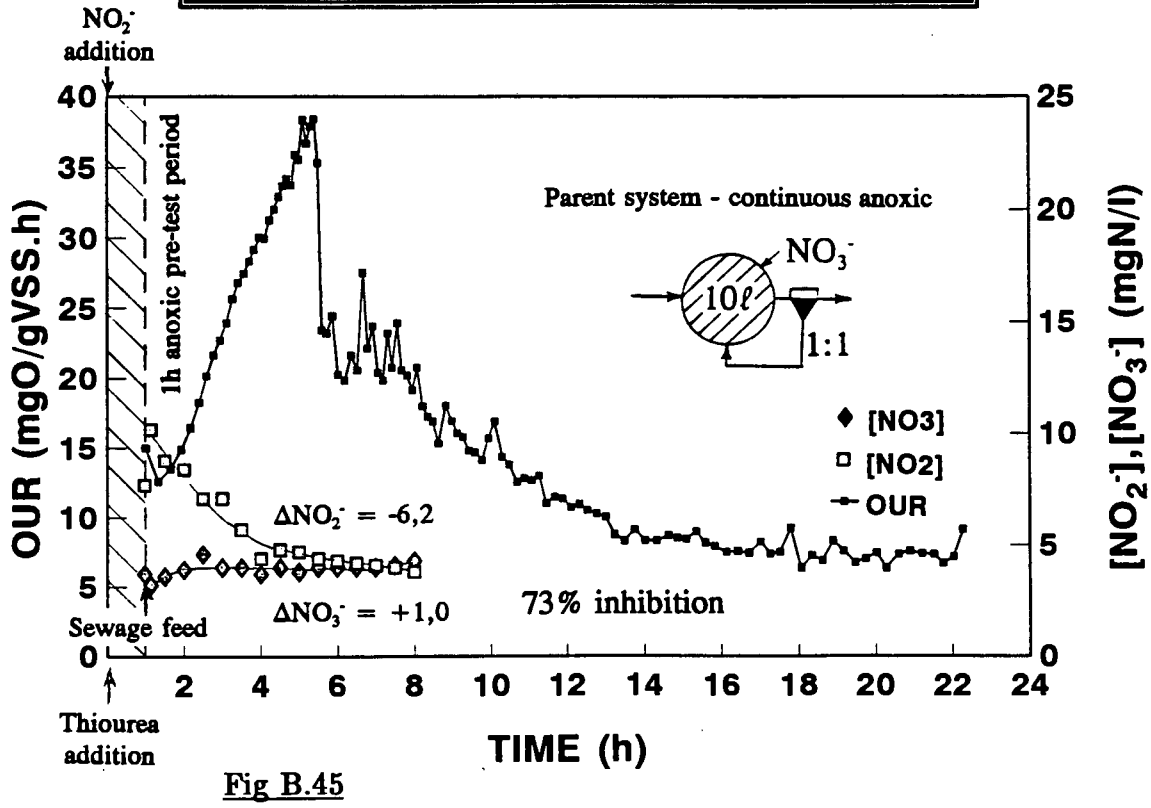


Fig B.45

Table B.69

BATCH TEST I OPERATING CONDITIONS		
Objective: To examine the effect of *aerobic* conditions on synthesis of denitrifying enzymes through analysis of inhibition of OUR due to accumulated nitric oxide (NO).		
PARENT SYSTEM CONDITIONS		
System 7; Day 333 **System configuration: Continuous aerobic**		10ℓ 1:1 0.2 < DO < 2.0
Sludge age	**(d)**	**15**
BATCH TEST CONDITIONS		
Sludge volume	**(ℓ)**	**1,6**
Water volume	**(ℓ)**	**0,4**
Sewage volume	**(ℓ)**	**1,0**
Total volume	**(ℓ)**	**3,0**
MLSS	**(mg/ℓ)**	**596**
VSS	**(mg/ℓ)**	**447**
F/M	**(mgCOD/mgVSS)**	**1,0**
Anoxic pre-test period	**(h)**	**-**
Aerobic pre-test period	**(h)**	**2,0**
DO	**(mgO/ℓ)**	**2 - 5**
Temperature	**(°C)**	**20**
SEWAGE TO BATCH TEST		
COD	**(mgCOD/ℓ-final batch vol)**	**436**
TKN	**(mgN/ℓ-final batch volume)**	**31,2**

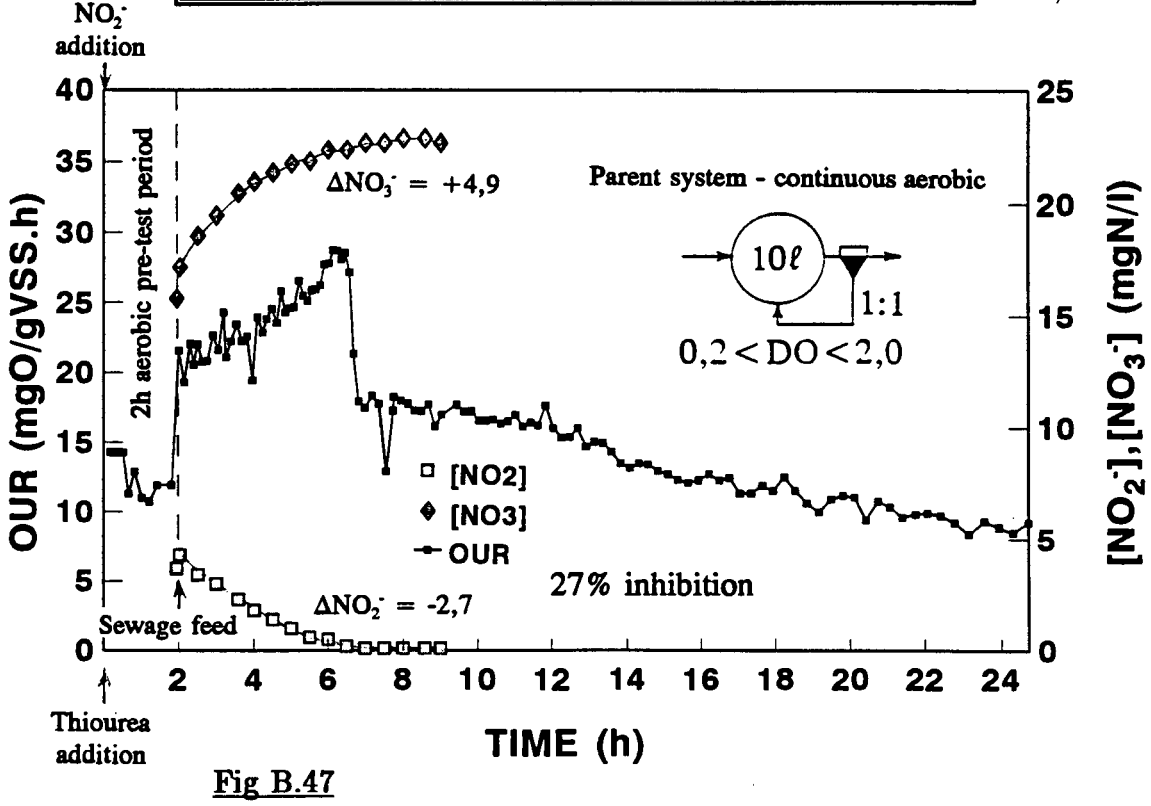
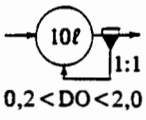


Fig B.47

Table B.71

BATCH TEST K OPERATING CONDITIONS		
Objective: To examine the effect of *aerobic* conditions on synthesis of denitrifying enzymes through analysis of inhibition of OUR due to accumulated nitric oxide (NO).		
PARENT SYSTEM CONDITIONS		
System 7; Day 353 System configuration: Continuous aerobic		
Sludge age	(d)	15
BATCH TEST CONDITIONS		
Sludge volume	(ℓ)	1,0
Water volume	(ℓ)	1,0
Sewage volume	(ℓ)	1,0
Total volume	(ℓ)	3,0
MLSS	(mg/ℓ)	449
VSS	(mg/ℓ)	393
F/M	(mgCOD/mgVSS)	1,2
Anoxic pre-test period	(h)	-
Aerobic pre-test period	(h)	2,0
DO	(mgO/ℓ)	2 - 5
Temperature	(°C)	20
SEWAGE TO BATCH TEST		
COD	(mgCOD/ℓ-final batch vol)	473
TKN	(mgN/ℓ-final batch volume)	36,8

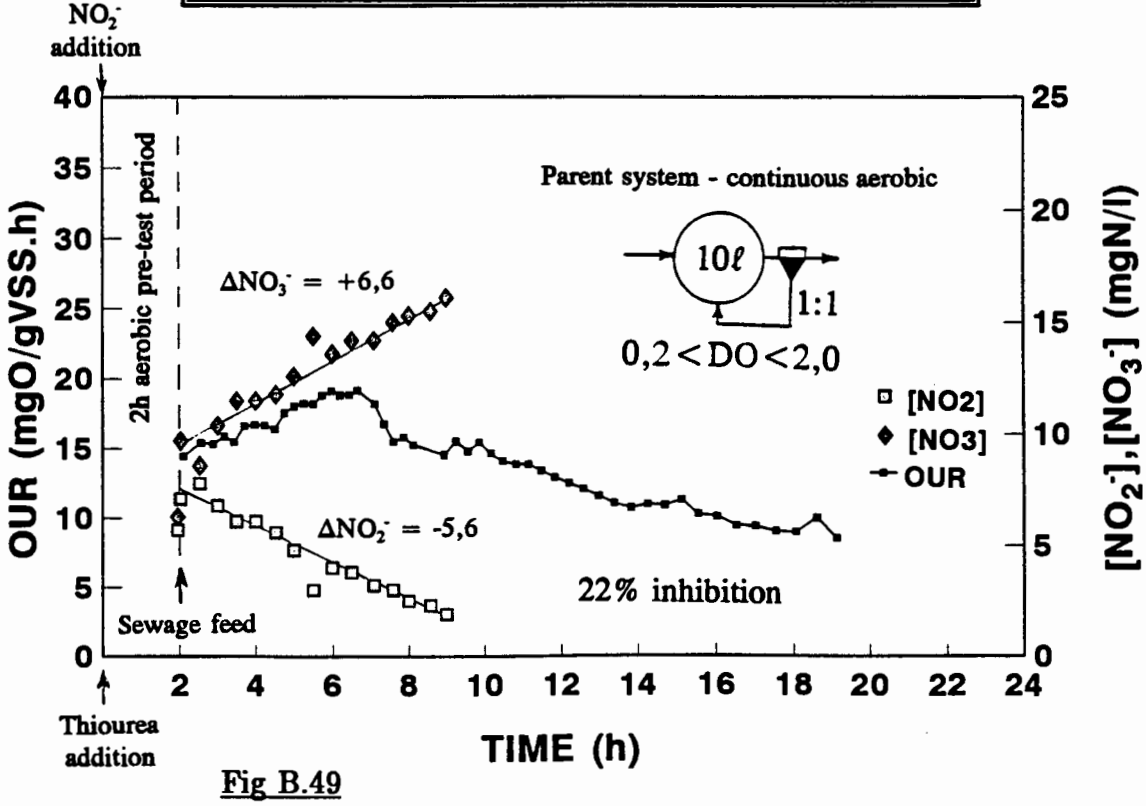


Fig B.49

Table B.73

BATCH TEST M OPERATING CONDITIONS		
Objective: To examine the effect of *anoxic* conditions on synthesis of denitrifying enzymes through analysis of inhibition of OUR due to accumulated nitric oxide (NO).		
PARENT SYSTEM CONDITIONS		
System 7; Day 354 System configuration: Continuous anoxic		
Sludge age	(d)	15
BATCH TEST CONDITIONS		
Sludge volume	(ℓ)	1,0
Water volume	(ℓ)	1,0
Sewage volume	(ℓ)	1,0
Total volume	(ℓ)	3,0
MLSS	(mg/ℓ)	496
VSS	(mg/ℓ)	444
F/M	(mgCOD/mgVSS)	1,1
Anoxic pre-test period	(h)	1,0
Aerobic pre-test period	(h)	-
DO	(mgO/ℓ)	2 - 5
Temperature	(°C)	20
SEWAGE TO BATCH TEST		
COD	(mgCOD/ℓ-final batch vol)	473
TKN	(mgN/ℓ-final batch volume)	36,8

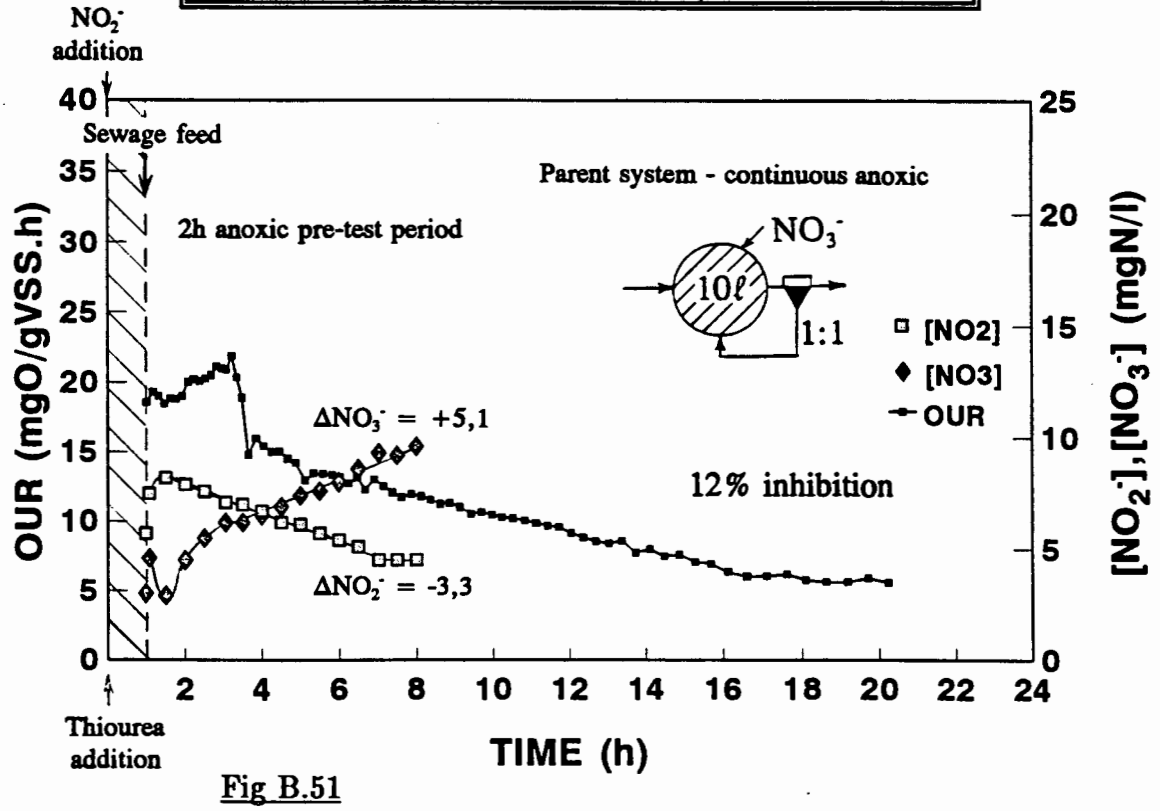
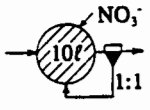


Fig B.51

Table B.75

BATCH TEST O OPERATING CONDITIONS		
Objective: To examine the effect of *anoxic* conditions on synthesis of denitrifying enzymes through analysis of inhibition of OUR due to accumulated nitric oxide (NO).		
PARENT SYSTEM CONDITIONS		
System 7; Day 356 System configuration: Continuous anoxic		
Sludge age	(d)	15
BATCH TEST CONDITIONS		
Sludge volume	(ℓ)	1,2
Water volume	(ℓ)	1,0
Sewage volume	(ℓ)	0,8
Total volume	(ℓ)	3,0
MLSS	(mg/ℓ)	427
VSS	(mg/ℓ)	388
F/M	(mgCOD/mgVSS)	0,8
Anoxic pre-test period	(h)	1,0
Aerobic pre-test period	(h)	1,0
DO	(mgO/ℓ)	-
Temperature	(°C)	2 - 5 20
SEWAGE TO BATCH TEST		
COD	(mgCOD/ℓ-final batch vol)	323
TKN	(mgN/ℓ-final batch volume)	31,9

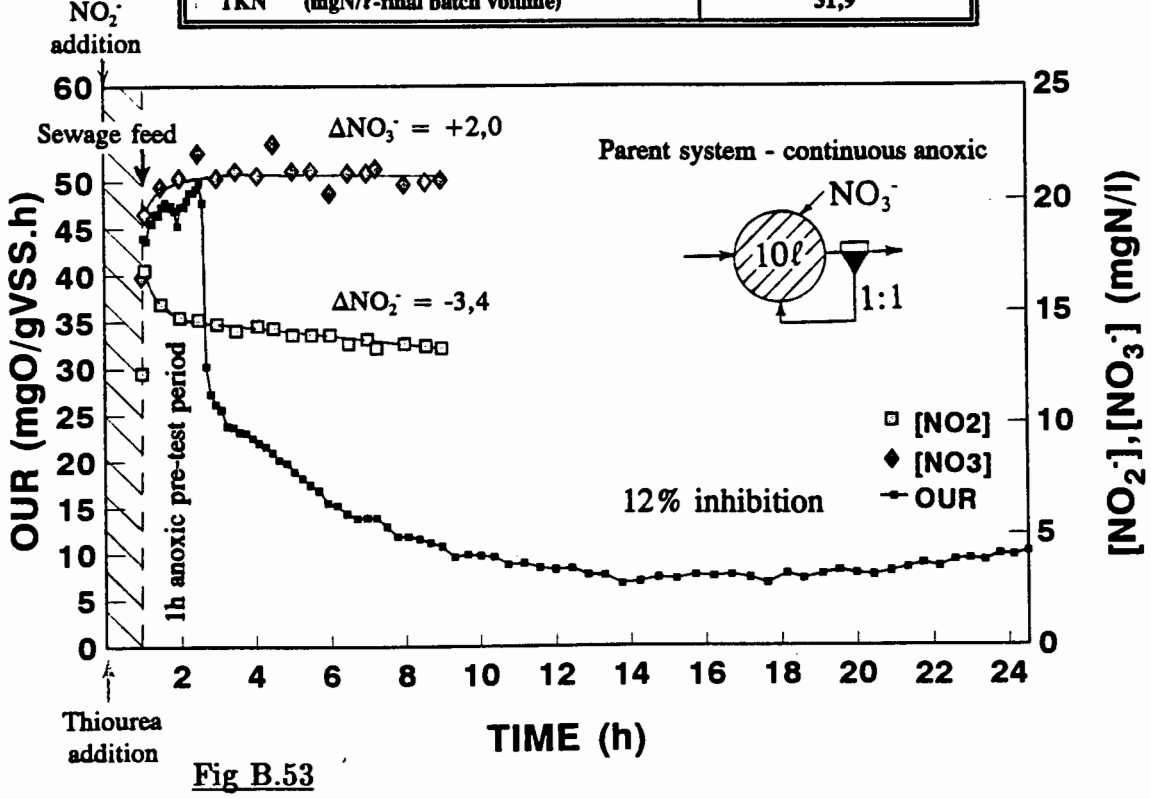
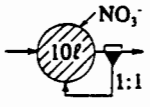


Fig B.53

Table B.77

BATCH TEST Q OPERATING CONDITIONS		
Objective: To examine the effect of *anoxic* conditions on synthesis of denitrifying enzymes through analysis of inhibition of OUR due to accumulated nitric oxide (NO).		
PARENT SYSTEM CONDITIONS		
System 7; Day 373 System configuration: Continuous anoxic		
Sludge age	(d)	15
BATCH TEST CONDITIONS		
Sludge volume	(ℓ)	1,5
Water volume	(ℓ)	0,5
Sewage volume	(ℓ)	1,0
Total volume	(ℓ)	3,0
MLSS	(mg/ℓ)	696
VSS	(mg/ℓ)	581
F/M	(mgCOD/mgVSS)	0,9
Anoxic pre-test period	(h)	1,0
Aerobic pre-test period	(h)	2,0
DO	(mgO/ℓ)	2 - 5
Temperature	(°C)	20
SEWAGE TO BATCH TEST		
COD	(mgCOD/ℓ-final batch vol)	423
TKN	(mgN/ℓ-final batch volume)	33,8

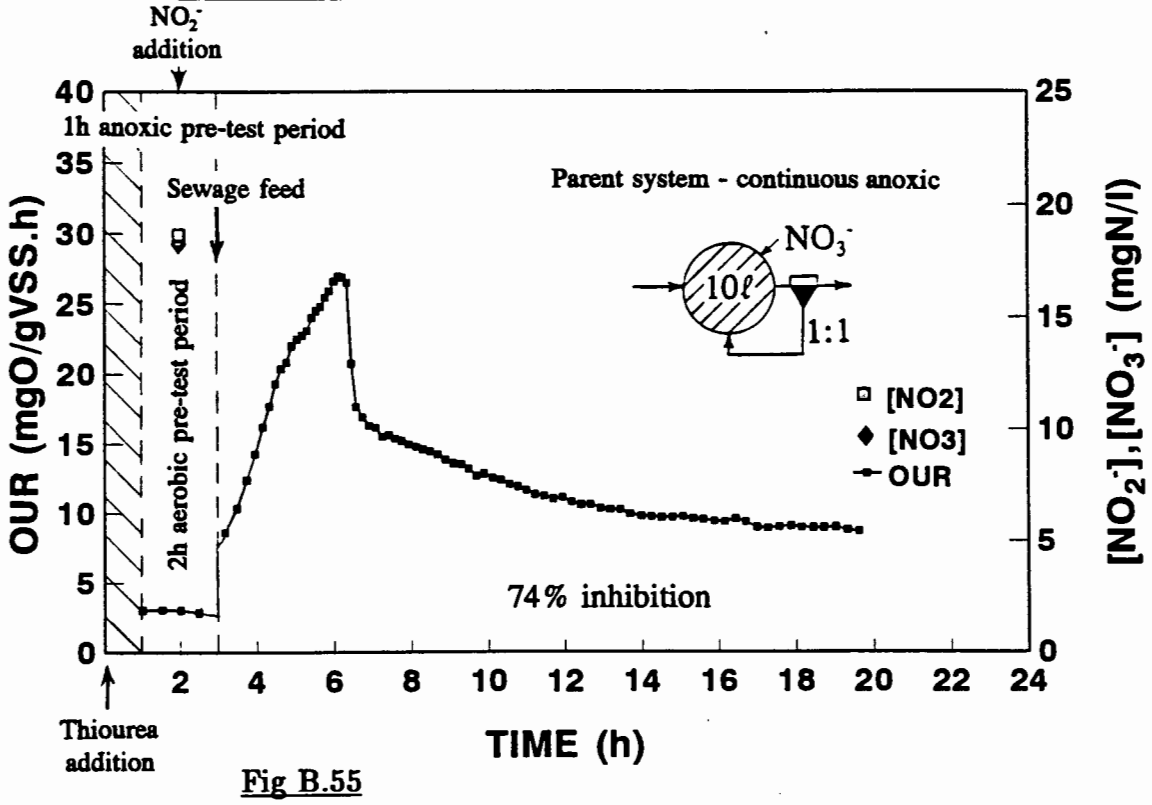


Fig B.55

Table B.79

DENITRIFICATION BATCH TEST 1 OPERATING CONDITIONS		
Objective: To determine the effect on the denitrification rate of a long period of exposure to aerobic conditions.		
PARENT SYSTEM CONDITIONS		
System 7 ; Day 350 System configuration: Continuous aerobic		10ℓ, 1:1, 0,2 < DO < 2,0
Sludge age	(d)	15
BATCH TEST CONDITIONS		
Sludge volume	(ℓ)	2,3
Water volume	(ℓ)	0,3
Sewage volume	(ℓ)	0,7
Total volume	(ℓ)	3,0
MLSS	(mg/ℓ)	1237
VSS	(mg/ℓ)	1041
F/M	(mgCOD/mgVSS	0,3
Temperature	(°C)	20
SUBSTRATE TO BATCH TEST		
COD	(mgCOD/ℓ-final batch vol)	327

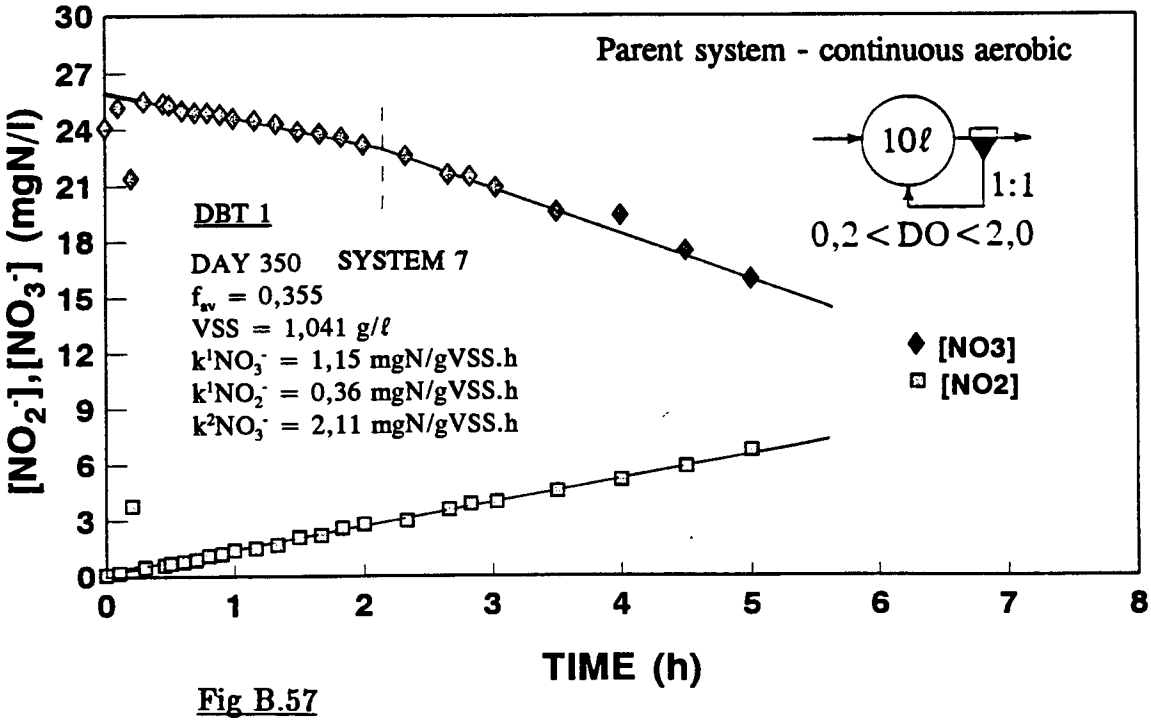
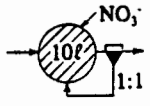


Fig B.57

Table B.81

DENITRIFICATION BATCH TEST 3 OPERATING CONDITIONS		
Objective: To determine the effect on the denitrification rate of a change from steady-state aerobic conditions to anoxic conditions.		
PARENT SYSTEM CONDITIONS		
System 7 ; Day 355 System configuration: Continuous anoxic		
Sludge age	(d)	15
BATCH TEST CONDITIONS		
Sludge volume	(ℓ)	2,0
Water volume	(ℓ)	0,3
Sewage volume	(ℓ)	0,7
Total volume	(ℓ)	3,0
MLSS	(mg/ℓ)	857
VSS	(mg/ℓ)	654
F/M	(mgCOD/mgVSS	0,5
Temperature	(°C)	20
SUBSTRATE TO BATCH TEST		
COD	(mgCOD/ℓ-final batch vol)	321

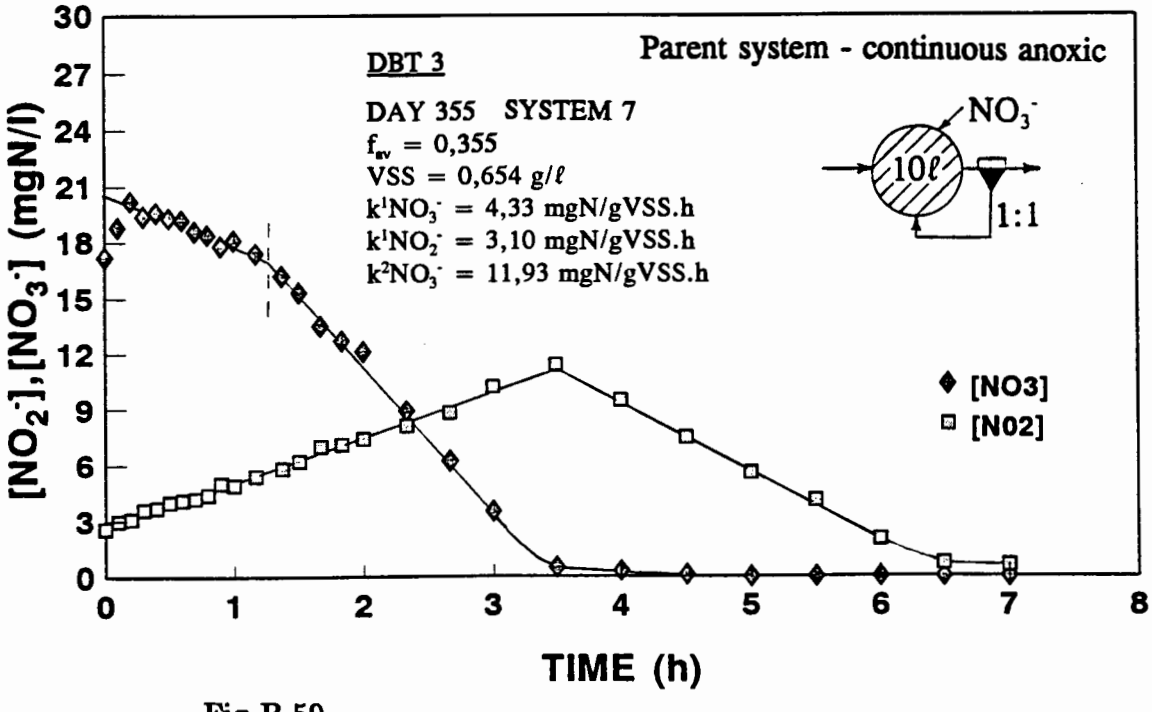


Fig B.59

APPENDIX B.5

ULTRAFILTRATION OF MUNICIPAL SEWAGE

As a means of obtaining the readily biodegradable and slowly biodegradable fractions of municipal sewage, (unsettled raw sewage from Mitchell's Plain treatment works, Cape Town) was subjected to ultrafiltration. Fig B.61 illustrates the experimental setup by which the sewage was fractionated. A set of typical experimental data illustrating the COD of the influent and filtrate during ultrafiltration and the portion of each of those fractions which passes 0,45μm is illustrated in Fig B.62.

APPENDIX C

A REVIEW OF RESEARCH INTO ELECTRON TRANSPORT PATHWAYS OF FACULTATIVE HETEROTROPHIC ORGANISMS

This review describes the research findings associated with identification of the structure, position with relation to the cytoplasmic membrane, and reactive mechanisms of the terminal electron transferring complexes, the nitrogen oxide reductases and the cytochrome oxidases of the electron transport pathway. From the research findings, an electron transport pathway is proposed as the one most representative of facultative heterotrophic organisms and is used throughout the thesis as such.

C.1 A METHODOLOGY FOR RESEARCH

In elucidating the biochemical pathways employed by an organism, it is often useful to compare the physiology of the organism being studied with another functionally similar organism, the physiology of which is well established. This experimental methodology was followed by workers investigating the biochemical pathways of denitrifying organisms. To digress briefly; heterotrophic organisms found in activated sludge are categorized according to their respiratory function (that is, obligate aerobic, (O_2 as electron acceptor), facultative aerobic, (O_2 or NO_3^-/NO_2^- as electron acceptors), or anaerobic, (electron acceptor internally generated). Single cell organisms which have a low level of biochemical pathway organization and derive their energy from external electron donors are known as *prokaryotes.* Organisms with a considerably higher level of biochemical pathway organization are known as *eukaryotes*, and in their cellular composition have a nucleus, separated from the cytoplasm by a nuclear membrane and more importantly as far as this work is concerned, a mitochondrion (i.e. an internal energy generating organelle). A further difference between prokaryotes and eukaryotes is that prokaryotes can be facultative whereas eukaryotes are obligate aerobes. Thus, facultative heterotrophic organisms found in activated sludge are regarded as prokaryotes. The biochemistry of the aerobic respiratory pathways of the mitochondrion of eukaryotic organisms is well established. A cursory examination of the aerobic respiratory pathways of prokaryotic organisms indicates many similarities to mitochondrial pathways, especially with regard to the enzymatic components of the aerobic respiratory pathway.[1] As a consequence of the metabolic similarities of prokaryotes and eukaryotes, the well established biochemistry of the mitochondrial respiratory pathways has been used as a model for research into the aerobic respiratory pathways of the prokaryotic organisms.

A number of organisms have been selected as representative of the prokaryotic group and research has been concentrated on these. In this regard there are two facultative organisms that have received considerable research attention;

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[1] The similarities between prokaryotes and eukaryotes have given rise to what is known as the endosymbiotic theory for the evolutionary origin in the eukaryotic organism (Margulis, 1970). According to this theory, an aerobic bacterium evolved to a mitochondrion. A simple aerobic prokaryotic bacterium which could oxidize organic substrate with oxygen as electron acceptor, merged with an amoeboid cell which depended on fermentation for production of energy; the metabolism of the two organisms eventually became integrated, giving rise to the eukaryotic organism. The adaptive denitrifying components of the aerobic bacterium are considered to be a subsequent evolutionary feature.

nitrous oxide or dinitrogen (denitrification). The respiratory pathways exhibited by these organisms embrace the major aerobic/anoxic respiratory pathways of facultative organisms in nitrogen removal activated sludge systems and the physiology of the respiratory biochemical pathways exhibited by each of these organisms is considered typical of the group to which each belongs. For this reason these organisms often were used in experimental work enquiring into the electron transport pathways of facultative heterotrophic organisms.

C.2 CONSTITUTIVE AND ADAPTIVE PATHWAY COMPONENTS

The respiratory pathways of the facultative organisms *Pa. denitrificans* and *E. coli* and the obligate aerobic mitochondrion in the eurkaryotes are similar both in function and components, but as noted earlier there is one notable difference. The mitochondrion is able to respire under aerobic conditions only, whereas *Pa. denitrificans* and *E. coli* can respire under aerobic and also under anoxic conditions with nitrate present. According to John and Whatley (1975), the components of the respiratory pathways of *Pa. denitrificans* and the mitochondrion which are similar and which develop under essentially all environmental conditions are: NADH, flavoprotein, iron–sulphur proteins, ubiquinone, cytochromes of the *b* and *c*–type and cytochrome aa_3. These are all components of the respiratory chain, but only some are enzymes. All these components are referred to by John and Whatley (1975) as *constitutive* components of the respiratory pathway, a reference to their presence under all environmental conditions. The difference between the respiratory pathways of *Pa. denitrificans* and a mitochondrion is the presence of the nitrogen oxide reductases in *Pa. denitrificans* which are not found in mitochondria. These are referred to as *adaptive* components, viewed by John and Whatley (1975) as *ad hoc* additions to the constitutive aerobic respiratory pathway, their development a consequence of growth of aerobic organisms under anoxic conditions.

In this thesis, considerable attention is devoted to the nature of the aerobic and denitrifying enzymatic components of the respiratory pathway, especially in determining whether these enzymes are constitutive or adaptive and the effect of oxygen or lack of oxygen on their formation and activity.

In microbiological work, enzymes that are present in nearly constant amounts under all environmental conditions are called *constitutive enzymes.* Enzymes which are adaptive by nature and are synthesized only in response to the presence of certain substrates (and electron acceptors) are usually called *inducible enzymes.* For both

The early research

A major contribution to research into the products of denitrification resulted from research conducted in the 1940's–1950's when workers extracted enzymes from cells of denitrifying organisms which after purification formed gaseous nitrogen oxides from the reduction of nitrate and nitrite *in vitro*. These enzymes, and those of the aerobic respiratory pathway had first been documented by MacMunn in 1884 after the discovery that cells contain iron containing (haem) pigments, known today as cytochromes. The leading biochemists of the time dismissed these observations as experimental error and it was not until the early 1920's that Kielin, while examining a suspension of yeast under a microscope viewed a four–banded absorption spectra representing three different pigments (the fourth band being common to all) and proper recognition was accorded MacMunn's work. Kielin realized that these substances acted as catalysts in the oxidation process and named them cytochromes *a*, *b* and *c*, the alphabetical terminology still being used today. The intervening years to the 1940's saw development in techniques for the extraction and purification of all the respiratory pathway enzymes.

Workers studying nitrate reduction in the 1940's–1950's extracted nitrate reducing fractions from *E. coli*, other organisms and also from green plants (Sato 1956; Nason and Evans 1953; Nicholas and Nason 1957; Taniguchi *et al.*, 1956). Two nitrate reductases were obtained, referred to as Pichinoty's nitrate reductase 'B' which served an assimilatory function (i.e. assimilation of nitrogen into cellular matter) and Pichinoty's nitrate reductase 'A', an enzyme with a dissimilatory function (i.e. energy production). *The dissimilatory enzyme only is of interest in this review.*

Taniguchi and Itagaki (1960) were the first to characterize a dissimilatory nitrate reducing enzyme, that of *E. coli*, grown anoxically in the presence of nitrate. It was found to contain 1 atom of bound molybdenum and 40 atoms of bound iron. Subsequently, other investigators measured similar compositions for the nitrate reductase from *E. coli* (MacGregor *et al.*, 1974; Forget, 1974). Regarding denitrifying organisms, Lam and Nicholas (1969b) purified a nitrate reductase from *Micrococcus denitrificans* (now *Pa. denitrificans*) shown by isotopic labelling to contain molybdenum but no flavin or cytochrome and Forget (1971) used chemical determinations on nitrate reductase from the same organism to yield an enzyme containing 0,4 atoms of molybdenum (Mo) and 8 atoms of iron (Fe) per molecule. The involvement of molybdenum and iron in the nitrate reductase of *Pa. denitrificans* was further verified by Forget and DerVartanian (1972), using

divergence of the electron pathway to nitrate reduction from the aerobic respiratory pathway. This aspect is highlighted in the following review of the development of the respiratory electron transfer pathways of facultative organisms.

Point of divergence from aerobic pathways of electron flow to nitrate reductase

As a means of determining the cytochromes of the respiratory pathway from which electrons pass to nitrate reductase, most workers adopted a policy of growing the organisms under 3 sets of conditions; aerobic, anoxic (nitrate present), and anaerobic (nitrate and O_2 absent). A conclusion regarding the effect of environmental conditions on enzyme synthesis was made by comparing the type and level of cytochromes produced under each set of conditions, based on the hypothesis that the enzyme complexes which exhibit the greatest development are those which are the most immediate to the electron acceptors, nitrate and oxygen.

In one of the first electron transfer schemes published for facultative organisms, illustrated in Fig C.1, Fewson and Nicholas (1961b) proposed that in *Pseudomonas aeruginosa* the aerobic and nitrate reducing electron transport pathways branch from cytochrome *c*, because anoxically grown (with nitrate present) *Ps. aeruginosa* exhibited an increase in cytochrome *c* concentration and nitrate reductase activity. Although cytochrome *b* was present in the enzymes, Fewson and Nicholas (1961b) were reluctant to assign an electron transferring function to this component, and cited evidence against its involvement in either aerobic or anoxic respiration. Because all the electron transferring complexes of the pathway were considered to be common to aerobic and anoxic respiration, there was no need to consider the constitutive or adaptive nature of the aerobic and anoxic components of the respiratory pathway. Molybdenum (Mo) was found to be a constituent of the nitrate reductase system, and it was suggested that during nitrate reduction, the molybdenum atom undergoes a valency change, probably $Mo^{6+} \rightarrow Mo^{5+}$. With respect to the effect of anoxic conditions on the development of cytochrome oxidase, when cells of *B. stearothermophilus* were changed from aerobic to anoxic growth conditions, with nitrate present, oxygen respiration decreased by between 50 and 70%; the cytochrome spectra indicating the disappearance of cytochrome a_3 (Downey, 1966; Downey *et al.*,1969). This indicated that cytochrome a_3 is an inducible rather than a constitutive enzyme and led to the consideration that another cytochrome oxidase, a constitutive enzyme is synthesized under anoxic conditions which allows respiration to occur immediately upon the introduction of oxygen.

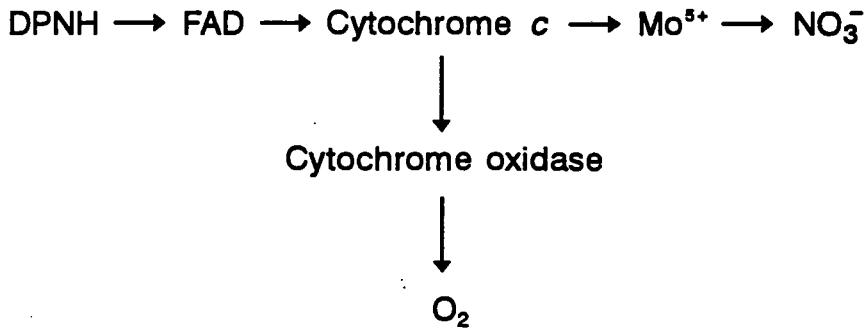

Fig C.1: Electron transport pathway (ETP) proposed by Fewson and Nicholas (1961b), for *Pseudomonas aeruginosa* in which cytochrome *c* is the branch point for electrons to nitrate reductase and cytochrome oxidase. [Abbreviations: DPNH – diphosphopyridine nucleotide (reduced); FAD – Flavin adenine dinucleotide; Mo – molybdenum.]

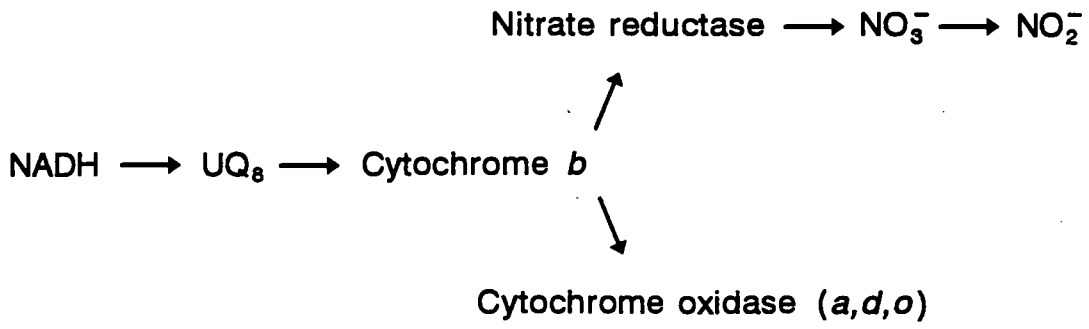

Fig C.2: Electron transport pathway (ETP) proposed by Knook *et al.* (1973) for *Klebsiella* (formerly *Aerobacter) aerogenes* in which cytochrome *b* is the branch point for electrons to nitrate reductase and cytochrome oxidase. [Abbreviations: NADH – nicotinamide adenine dinucleotide (reduced); UQ_8 – ubiquinone 8.]

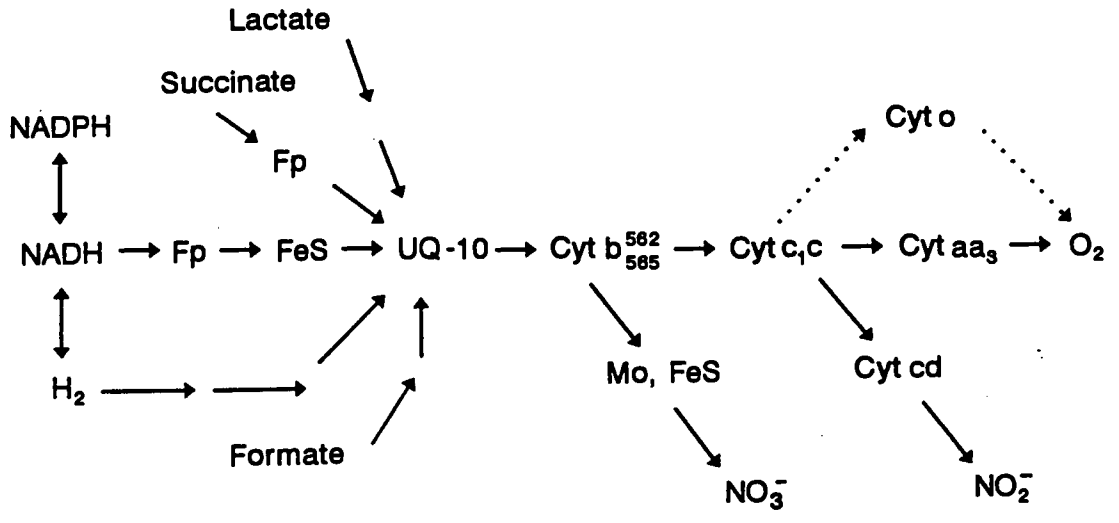

Fig C.3: Electron transport pathway (ETP) proposed by John and Whatley (1975) for *Paracoccus denitrificans* in which cytochrome *c* complex is the branch point for electrons to nitrite reductase and the oxidase cytochrome aa_3, cytochrome *b* is the branch point to nitrate reductase and an alternative oxidase, cytochrome *o* has a branch point from cytochrome *c*. [Abbreviations: NADPH – nicotinamide adenine dinucleotide phosphate (reduced); Fp – flavoprotein; FeS – iron sulphur centres; UQ–10 – ubiquinone 10; Cyt– Cytochrome; Mo – Molybdenum.]

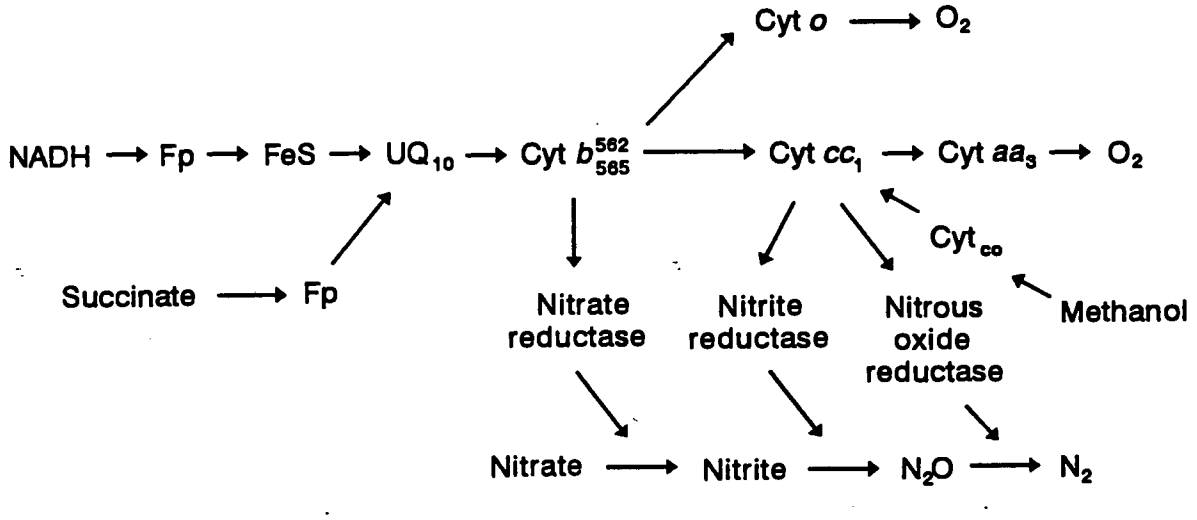

Fig C.4: Electron transport pathway (ETP) proposed by Stouthamer (1980) for *Paracoccus denitrificans* in which nitrous oxide reductase is included with an electron branch point from cytochrome cc_1. [Abbreviations: NADH – nicotinamide adenine dinucleotide (reduced); Fp – flavoprotein, FeS – iron sulphur centres; UQ_{10} – ubiquinone 10; Cyt – Cytochrome.]

points of divergence to nitrate reductase and cytochrome *o* are at ubiquinone, which is shown as a homogeneous pool connecting the dehydrogenases with the cytochromes. Changing the point of divergence of electrons to nitrate reductase from cytochrome *b* to ubiquinone resulted from work conducted by Alefounder *et al.* (1981) with the inhibitory chemical antimycin A. The mode of action of inhibitors of the electron transport pathway is to restrict the flow of electrons between specific cytochromes. The point of action of antimycin A has conventionally been allocated to between the cytochrome *b* and cytochrome *c* complexes (Knobloch et al., 1971). However, the exact point of action of antimycin A is somewhat unclear owing to the schematic separation of cytochromes of the *c* complex (i.e. cytochrome *c* and cytochrome c_1) and the organization of cytochromes *b* and c_1 into one complex. The work of Alefounder et al., (1981) indicates that antimycin A restricts electron flow through the cytochrome bc_1 complex and as a result of the finding that antimycin A does not reduce the rate of nitrate reduction by *Pa. denitrificans* (John and Whatley, 1975), Ferguson (1982) proposed ubiquinone as the point of divergence to nitrate reductase. This proposal was supported by Kučera *et al.* (1984b) working with *Pa. denitrificans* and the inhibitors antimycin A and mucidin. They suggested that the observations of Lam and Nicholas (1969a) and John and Whatley (1970) that cytochrome *b* of the cytochrome bc_1 complex is oxidized following the addition of nitrate, is a consequence of an increase in the degree of ubiquinone reduction due to the flow of electrons from ubiquinone to the nitrate reductase complex. Kučera *et al.* (1984b) suggested that a more likely explanation for the oxidation of cytochrome *b* during nitrate reduction is that *nitrate reductase contains cytochrome b*, a suggestion motivated by analogy with the constituents of the nitrate reductase of *E. coli* (Ferguson, 1982) and by work conducted by Calder and Lascelles (1984) with *Pa. denitrificans.*

Experimental evidence supporting the organization of respiratory components into complexes in the manner of Ferguson (1982) was furnished by Berry and Trumpower (1985). They isolated from *Pa. denitrificans* a ubiquinol oxidase, containing a cytochrome bc_1 complex, and a cytochrome c-aa_3 complex. The complexes corresponded to those of the mitochondrial complexes, even to the point that inhibitors of electron flow through the mitochondrial bc_1 complex inhibited electron flow through the same complex of *Pa. denitrificans*, indicating the similarity in the pathways. Further work by Yang and Trumpower (1986) on the electron transferring function of the cytochrome bc_1 complex of *Pa. denitrificans* indicated that it is similar if not identical to the cytochrome bc_1 complex of

oxidized side of the electron transport pathway to nitrate reductase, the reason for its increase under anoxic conditions in the presence of nitrate was perplexing and needed to be addressed. From work conducted on *E. coli*, Ruiz–Herrera and De Moss (1969) proposed that a *b*–type cytochrome, distinct from that involved in the aerobic electron transport pathway, functions as part of the nitrate reductase complex under anoxic conditions with nitrate present. Further support for this explanation was gained by workers examining the structure of nitrate reductase and the role of its subunits in catalyzing electron flow, and is discussed below.

Variation in the molecular weights reported for nitrate reductase result from different extraction and purification techniques (Stouthamer, 1976). These different techniques led also to different numbers of subunits being reported for nitrate reductase, not only between organisms, but also from the same organism (Hochstein and Tomlinson, 1988). Because of the important catalytic role of nitrate reductase, the composition and function of this enzyme is of considerable importance and it is imperative that the number and function of these subunits is well established in order to understand the mechanisms of electron transfer to nitrate.

The nitrate reductases of many denitrifying organisms are reported to consist of three polypeptides, referred to as subunits α, β and γ (*K. aerogenes*, van 't Riet and Planta, 1975; *Proteus mirabilis*, Oltmann *et al.*, 1976a; *B. licheniformis*, van 't Riet *et al.*, 1979; *Ps. denitrificans*, Ishizuka *et al.*, 1984; and *Pa. denitrificans*, Craske and Ferguson 1986). However, initial work on characterizing this enzyme from some denitrifying organisms had detected only two polypeptides, referred to as subunits α and β (*B. licheniformis* Weintjes *et al.*, 1979; *K. aerogenes*, Weintjes *et al.*, 1979, Abraham *et al.*, 1981; *Ps. aeruginosa*, Carlson *et al.*, 1982).

In a similar fashion, the nitrate reductase of *E. coli* was also initially reported as having two subunits (MacGregor *et al.*, 1974) and then later three subunits (Enoch and Lester, 1974; MacGregor 1975b; Morpeth and Boxer, 1985), the difference being attributed to the method used to release the membrane bound enzyme (Enoch and Lester, 1975; MacGregor, 1976; Lund and de Moss, 1976).

It was established that the number of nitrate reductase subunits detected from denitrifying organisms is also dependent on the extraction procedure used (Craske and Ferguson, 1986; Ishizuka *et al.*, 1984; van't Riet *et al.*, 1979) These workers suggested that the use of heat treatment extraction procedures as opposed to

with the membrane (MacGregor, 1975c). It catalyzes the transfer of electrons from ubiquinol to the α subunit (Ingledew and Poole, 1984). Chaudhry and MacGregor (1983a) established that the iron–sulphur (FeS) and molybdenum centres of nitrate reductase in this organism are associated with the α subunit, and since EPR studies have implicated these centres in electron transfer, the catalytic site of the reductase is allocated to subunit α. The role of subunit β is not as well understood. Some work suggests that it is involved in membrane attachment (Ingledew and Poole, 1984; Stouthamer, 1988; Stewart, 1988), or that it has a role in regulating the synthesis of nitrate reductase (Ingledew and Poole, 1984), but it does not appear to be involved in electron transfer between subunits α and γ.

Stewart (1988), in a review of the work of MacGregor (1976), Stewart and MacGregor (1982) and Hackett and MacGregor (1981), concludes that subunits α and β are synthesized as soluble precursors (i.e. not bound to the membrane) to nitrate reductase formation and that the presence of subunit γ is essential for assembly of the reductases into the cytoplasmic membrane. (For the effect of oxygen on the assembly of the subunits into the membrane, see Appendix D).

Less work has been conducted on the subunits of nitrate reductase in denitrifying organisms than has been conducted on the subunits of nitrate reductase in the nitrate reducing organism *E. coli*. Craske and Ferguson (1986) suggest a mechanism for electron flow through nitrate reductase of *Pa. denitrificans* based on their own findings and also by analogy with the flow of electrons through the nitrate reductase of *E. coli* (Ingledew and Poole, 1984).

> "..... in the plasma membrane of *P. denitrificans* the γ subunit catalyzes electron transfer to the α and β subunits of nitrate reductase from ubiquinol which acts as a branch point in the respiratory chain."

Continuing the analogy with nitrate reductase of *E. coli* in which the iron–sulphur clusters and molybdenum are associated with the α subunit (Chaudhry and MacGregor, 1983b) the catalytic site of nitrate reductase of *Pa. denitrificans* is similarly allocated to subunit α, due to the finding that two or three iron–sulphur clusters and molybdenum which are the active centres of nitrate reductase in *E. coli* are also found in various denitrifiers (*Pa. denitrificans*, Forget, 1971; *B. licheniformis*, van 't Riet *et al.*, 1979; and *K. aerogenes*, van 't Riet *et al.*, 1975).

(1983a) proposed two uptake systems for nitrate. The first system initiates nitrate uptake and is dependent on the membrane electrical potential, alternatively called proton motive force (pmf). Nitrate crosses the membrane together with two or more protons via an H^+/NO_3^- symport system and is reduced to nitrite at the catalytic site of nitrate reductase at the α subunit located on the cytoplasmic side. The second nitrate uptake system is an NO_3^-/NO_2^- antiport which takes over nitrate uptake from the first system. In an exchange mechanism, one nitrite molecule produced from nitrate reduction passes back to the periplasm for each nitrate molecule crossing the membrane to the cytoplasm. The process is electroneutral and self perpetuating so long as nitrate is present.

Although present evidence points to a NO_3^-/NO_2^- antiport as a likely mechanism for nitrate and nitrite transport, the evidence is circumstantial and no physical evidence of such a system has yet been demonstrated. Consequently, Craske and Ferguson (1986) suggest that the nitrate reductase complex itself incorporates a nitrate–specific channel which provides access for nitrate to the active site of its reductase. In conclusion, it is apparent that more work needs to be done to elucidate the mechanisms of nitrate transport across the membrane to nitrate reductase. The effect of factors such as oxygen on the movement of nitrate across the membrane is discussed in Appendix D.

Regarding the active site of nitrate reductase, the mechanism of the catalytic action of nitrate reductase and the role of each of the subunits of the reductase have been documented in far greater detail for the nitrate reducing organism *E. coli* than for denitrifying organisms. For *E. coli*, a proposal for the manner by which the molybdenum component of the nitrate reductase complex acts as a catalyst in the reduction of nitrate to nitrite by coupled proton–electron transfer was outlined by Stiefel (1973) and is illustrated in Fig C.8. Molybdenum (Mo) in oxidation state (IV) and attached to a fully protonated ligand interacts with nitrate. The Mo atom changes from oxidation state (IV) to (VI), with the concomitant reduction of NO_3^- by two electrons. The atom XH becomes acidic and transfers its proton to nitrate which then splits to nitrite and hydroxide. It is presumed that the Mo in the (VI) state is then reduced by electron transfer from the FeS clusters in the enzyme, to regain its Mo (IV) state.

The section above concludes the review of the function and position of the nitrate reductase and covered aspects such as (1) the development of the ETP with

emphasis on establishing the branch point of the electron flow to nitrate reductase, and (2) the structure of nitrate reductase and its orientation with respect to the cytoplasmic membrane, and (3) the catalytic action of the nitrate reductase in its electron transport function, and (4) mechanisms by which nitrate is transported across the membrane.

The end product of the catalytic action of nitrate reductase is nitrite and the following section reviews the next step in the denitrification chain, i.e. the enzymatic reduction of nitrite by the enzyme complex nitrite reductase.

C.4 NITRITE REDUCTASE

Research into the second step of the denitrification process, i.e. the reduction of nitrite, followed a pattern similar to that of research into the reduction of nitrate as outlined above. Nitrite reducing fractions were extracted from organisms respiring on nitrite under anoxic conditions and shown to have enzymatic characteristics *in vitro* with nitrite as electron acceptor. Extracts were purified and characterized, and the position on the electron transport chain at which electrons are passed to nitrite reductase was established. A major difference between research into nitrite reductase and nitrate reductase is that whereas the product of nitrate reduction (i.e. nitrite) was well established, the identity of the product of nitrite reduction (i.e. either nitrous oxide or nitric oxide) was the source of considerable argument. A further difference between research into the two reductases is that workers were able to draw an analogy between the nitrate reductase of *E. coli* and that of denitrifying organisms, but the absence of nitrite reduction in *E. coli* precluded this in an analysis of the nitrite reduction step of denitrifying organisms.

Respiratory nitrite reductase was first reported in cell free extracts of *Ps. aeruginosa* in 1939 by Yamagata. It has been found subsequently in *Thiobacillus denitrificans* (Baalsrud and Baalsrud, 1954), *Bacillus subtilus* (Najjar and Allen, 1954), *Ps. aeruginosa* (Yamanaka and Okunuki, 1963a), *Ps. stutzeri* (Chung and Najjar, 1956a), and *Ps. denitrificans* (Radcliffe and Nicholas, 1968).

Nitrite reductase was purified more than 600 fold from extracts prepared from actively reducing *P. aeruginosa*, by Walker and Nicholas (1961b) who identified a *c*-type cytochrome and another complex supposed to be a copper containing enzyme, the combination being able to reduce nitrite to nitric oxide.

conditions the possibility arises that both oxygen and nitrite could compete for nitrite reductase. However Payne (1981) indicates that the redox potential established at cytochrome oxidase precludes electron transfer to nitrite reductase.

Two types of nitrite reductase exist, the cytochrome *cd* complex discussed above and a copper–containing metallo–flavoprotein which also exhibits oxidase activity and has been isolated from *Achromobacter cycloclastes*, (Iwasaki and Matsubara, 1972; Iwasaki *et al.*, 1975), *Alcaligenes* sp. (Iwasaki *et al.*, 1963) and from strains of *Rhodospeudomonas sphaeroides*, (Sawada and Satoh, 1980).

Since no difference is apparent between these cytochromes, i.e. the *cd*–type and copper containing ones, in their activity, function, or position on the electron transport pathway, no distinction will be made between them when discussing the general function of nitrite reductase in the electron transport pathway, in the remainder of this review.

<u>Nitrite reduction product identification</u>

While agreement between groups was relatively uniform concerning characterization of the nitrite reductases for various denitrifying bacteria, an area of vigorous dispute emerged concerning the identity of the major product of the reduction of nitrite by nitrite reductase during denitrification, i.e. either nitric oxide (NO) or nitrous oxide (N_2O). This is of considerable importance since the identification of nitric oxide as a free obligatory intermediate would necessitate the existence of a specific nitric oxide reductase for its reduction.

The resolution of the question of whether nitric oxide is an intermediate was hampered by the finding that generally, nitric oxide production was detected from cell free extracts only, and not from whole cell suspensions (*Ps. stutzeri*, Najjar and Allen, 1954; *Bacillus licheniformis*, Najjar and Chung, 1956; *T. denitrificans*, Baalsrud and Baalsrud, 1954; *Ps. aeruginosa*, Walker and Nicholas, 1960). Some workers detected nitric oxide production in whole cell suspensions of denitrifying organisms (*Corynebacterium nephridii*, Renner and Becker, 1970; *Ps. denitrificans*, Radcliffe and Nicholas, 1968; *Pseudomonas perfectomarinus* – now *Pseudomonas perfectomarina*, Barbaree and Payne, 1967). This anomaly was partially resolved when Verhoeven (1956) demonstrated that whole cell suspensions of *Pa. denitrificans* and *Ps. stutzeri* did not produce extracellular nitric oxide under normal denitrifying conditions, but nitric oxide was produced by whole cell

(iii) Nitric oxide is a free intermediate between nitrite and nitrous oxide and is kinetically controlled to very low concentrations.

The Tiedje research group has favoured option (i). They assign the responsibility for the reduction of nitrite to nitrous oxide entirely to nitrite reductase. The scheme of Averill and Tiedje (1982) requires that nitrite traps an enzyme–bound nitroxyl ion (NO^+) to form bound N_2O_3 which is then reduced by two subsequent steps to nitrous oxide. These studies were conducted using Nitrogen–15 isotope labelling. Experiments using various isotopes of nitrogen were designed by the Tiedje group from which it was concluded that the hypothesized mechanism for nitrite reduction avoids the production of nitric oxide, (Aerssens *et al.*, 1986; Weeg–Aerssens *et al.*, 1987; 1988). (It is interesting that whereas this mechanism does not allow for the inclusion of NO as an intermediate, an early study by this group (Firestone *et al.*, 1979) using Nitrogen–13 isotope labelling concluded that nitric oxide did constitute part of the denitrification pathway, either as an intermediate or as a species in rapid equilibrium with an unidentified intermediate).

The complexity of determining the role of nitric oxide as an intermediate in denitrification, is highlighted by the efforts of the Hollocher group who over a number of years covered the spectrum of possible options. Initial experiments suggested that nitric oxide is not produced as an intermediate, i.e. option (i). Further experiments implicated nitric oxide as a membrane bound intermediate, i.e. option (ii). Later experiments confirmed NO as a free intermediate requiring its own reductase, i.e. option (iii). Details of these experiments and the organisms on which they were conducted are given below.

The majority of experiments conducted by this group used isotopically labelled Nitrogen–15 (^{15}N).

St John and Hollocher (1977) using gas chromatography and mass spectrometry to analyse isotopically labelled intermediates, found evidence for nitrous oxide but not nitric oxide as a free obligatory intermediate during denitrification of nitrite to dinitrogen by *Ps aeruginosa.*

Garber and Hollocher (1981) studied the role of nitric oxide in denitrification pathways in *Pa. denitrificans, Ps. stutzeri* and *Ps. aerofaciens* and ruled out the involvement of free nitric oxide as an obligatory intermediate in reduction of nitrite

The Payne group has consistently presented evidence in favour of option (iii). Payne *et al.* (1971) separated a nitrite–reducing complex from *Ps. perfectomarinus.* The specificity of the complex to nitrite reduction only was demonstrated by the fact that nitric oxide was the only gaseous product detected from the reduction of nitrite, and the complex failed to reduce nitrate, nitric oxide or nitrous oxide.

Even though the early evidence presented by the Payne group in favour of nitric oxide being a true intermediate appeared to be irrefutable, it was to a large extent disregarded by other workers. This lack of acceptance continued despite further evidence supplied by the Payne group in support of the formation of nitric oxide from nitrite reduction; Le Gall *et al.*, (1979) purified nitrite reductase from *T. denitrificans* which after reaction with nitrite formed nitric oxide.

In a number of reviews over the years, Payne has considered the evidence both for and against the formation of nitric oxide as a denitrification intermediate; (Payne, 1973; Payne, 1981), the conclusion in each case being that the weight of available evidence lends considerable support for nitric oxide formation from nitrite.

Similar evidence has come subsequently from many other research groups working with a great variety of organisms, e.g. *Ps. aeruginosa*, (Walker and Nicholas, 1961b; Yamanaka *et al.*, 1961; Shimada and Orii, 1975; Saraste and Kuronen, 1978; Kučera, 1989), *Pa. halodenitrificans*, (Grant and Hochstein, 1984), *T. denitrificans*, (Sawhney and Nicholas, 1978), *R. sphaeroides* forma sp denitrificans, (Sawada and Satoh, 1980), *Pa. denitrificans*, (Carr *et al.*, 1989; Carr and Ferguson, 1990b), *Ps. perfectomarina* (Zafiriou *et al.*, 1989).

Proper consideration of the available evidence suggests that nitric oxide should be assigned a role as a free intermediate in the denitrification chain, the only evidence which excludes it, being either hypothetical, or having been shown to be flawed by experimental artifact (i.e. ^{15}N isotope labelling techniques).

<u>Orientation of Nitrite Reductase</u>

Sawhney and Nicholas (1978) considered the nitrite reductase from *T. denitrificans* to be membrane bound, indicating that it is incorporated as a part of the membrane. However, they did not ascribe the reactive site of the reductase to either the periplasmic or cytoplasmic side of the membrane. Weintjes *et al.* (1979) also suggested nitrite reductase to be transmembrane in *K. aerogenes.*

extracted from *Ps. perfectomarinus* and that nitrous oxide was the only product detected from the reduction of nitric oxide and Pichinoty *et al.* (1979) isolated seven strains of *Bacillus* sp. which grew anoxically with nitric oxide as terminal electron acceptor.

Orientation of nitric oxide reductase

Zumft and Frunzke (1982) described a nitric oxide reductase from *Ps. perfectomarinus* as being membrane bound and able to catalyse the reduction of nitric oxide to nitrous oxide, but did not describe the side of the membrane on which the active site of the reductase is located.

Heiss *et al.* (1989), purified a nitric oxide reductase from *Ps. stutzeri* containing *b*– and *c*–type haem and allocated the active site to the cytoplasmic membrane and Hoglen and Hollocher (1989) purified and characterized a membrane–bound nitric oxide reductase from *Pa. denitrificans.* In a comprehensive study of nitric oxide reductase from *Pa. denitrificans*, Carr *et al.* (1989) were able to demonstrate that:

(i) Nitric oxide reductase is membrane bound, on the cytoplasmic side of the membrane.

(ii) Nitric oxide reductase is a discrete enzyme as opposed to being part of a nitrite reductase complex.

(iii) The addition of Triton X–100 as a means of selectively inhibiting reduction of nitric oxide, led to detection of nitric oxide upon the reduction of nitrate, but nitric oxide was not detected upon the reduction of nitrate in the absence of Triton X–100, indicating that under normal denitrifying conditions, steady state concentrations of nitric oxide are maintained at very low levels.

Subunit structure of nitric oxide reductase

Barbaree and Payne (1967) removed a sub–fraction rich in *c*–type cytochrome from the nitric oxide reducing fraction derived from *Ps. perfectomarinus* and the remaining fraction lost almost all capacity for reduction of nitric oxide. After return of the sub–fraction the capacity for nitric oxide reduction also returned.

Heiss *et al.* (1989) isolated a membrane–bound cytochrome *bc* complex from *Ps. stutzeri* which transformed nitric oxide to nitrous oxide. The enzyme complex

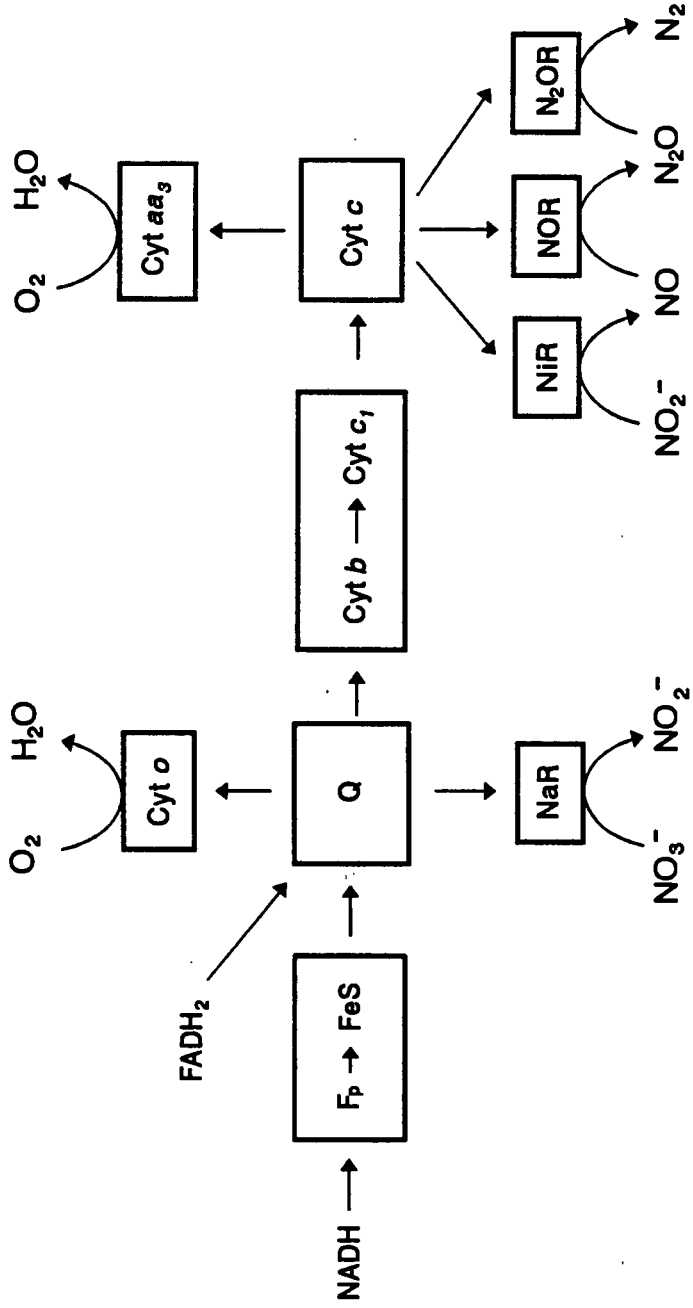

Fig C.9: Electron transport pathway (ETP) for facultative heterotrophic organisms in which the components are organized into complexes and nitric oxide reductase is included with the electron branch point from Cytochrome *c*. Adapted from Ferguson (1982). [Abbreviations: NADH – nicotinamide adenine dinucleotide (reduced); $FADH_2$ – flavin adenine dinucleotide (reduced); Fp – flavoprotein; FeS – iron sulphur centres; Q – ubiquinone; Cyt – Cytochrome; NaR – nitrate reductase; NiR – nitrite reductase; NOR – nitric oxide reductase; N_2OR – nitrous oxide reductase.]

anoxic respiration a second choice mechanism. However the intrinsic nature of the aerobic and anoxic components of the respiratory pathway means that it is impossible to examine anoxic respiration without also examining aerobic respiration. For this reason, the enzymes which function under aerobic conditions and comprise a part of the respiratory chain are also reviewed here. Of particular importance is the complex of enzymes known as cytochrome oxidase which acts to transfer electrons to the terminal electron acceptor oxygen.

C.7 CYTOCHROME OXIDASE IN FACULTATIVE ORGANISMS

Anoxic conditions which result in the synthesis of the denitrifying reductases also affect the synthesis and activity of the oxidases. In facultative organisms, a cytochrome oxidase complex additional to cytochrome aa_3 is present. This complex is not present in obligate aerobes, but is always present in facultative organisms, whether grown under aerobic or anoxic conditions, i.e. it is a constitutive complex. The presence of this complex defines the organism as having a facultative capacity. Because of the critical importance of this oxidase in facultative organism behaviour, it is necessary to review the research which led to its discovery and subsequent inclusion as part of the ETP for facultative organisms. Initial work concerned with the effect of anoxic conditions on cytochrome oxidase was directed entirely at cytochromes of the *a*–type since at that time it was considered that no other oxidase existed. It was considered that oxidase activity in facultative organisms resulted from the same enzymes as oxidase activity in obligate aerobic organisms.

Scholes and Smith (1968) working with *Pa. denitrificans* showed that the content of *a*–type cytochromes is considerably lower in organisms grown anoxically (with nitrate present) than in organisms grown aerobically.

Downey *et al.* (1969) investigating the effect of anoxic growth conditions on the aerobic respiration rate of *B. stearothermophilus* found that a culture grown under anoxic conditions increased their level of nitrate reductase 5 to 10–fold over an aerobically grown culture but did not decrease their aerobic respiratory activity proportionally, retaining 30 to 40% of the activity of aerobically grown cells, under subsequent aerobic conditions. This indicated either that nitrate reductase and cytochrome oxidase activity are controlled by separate mechanisms or that a constitutive aerobic enzyme with a lower activity is synthesized under anoxic conditions.

Stouthamer (1978) and Stouthamer (1980) offered evidence based on work conducted on *Pa. denitrificans* with the inhibitor antimycin A that the branching point for cytochrome *o* is not at cytochrome *c* but at cytochrome *b* (see Fig C.4). These workers considered that the site of action of antimycin A was between cytochrome *b* and cytochrome *c*, and that it halted electron flow between these two cytochromes. Ferguson (1982), argued against cytochrome *c* as electron donor to cytochrome *o*, one reason being that Willison and John (1979) had shown that a mutant of *Pa. denitrificans* which lacked cytochrome *c* still was able to grow aerobically, due to the presence of an alternative respiratory pathway terminating in a "cytochrome *o*–like oxidase". Ferguson (1982) also argued against cytochrome *b* as electron donor to cytochrome *o*, because electron flow to cytochrome *o* is insensitive to antimycin (Alefounder *et al.*, 1981) and antimycin is a powerful inhibitor of electron flow through the cytochrome bc_1 complex. For these reasons, ubiquinone was proposed as the immediate electron donor to cytochrome *o* and is indicated as such in Fig C.6.

The question then arises as to why different oxidases develop under different environmental conditions. For an organism subjected to alternating aerobic and anoxic periods, the production of the constitutive enzyme cytochrome *o* under anoxic conditions would permit it to immediately begin respiring aerobically after it has been subjected to prolonged anoxic periods (during which time the aerobically inducible enzyme cytochrome aa_3 is degraded to very low or insignificant levels). Because aerobic respiration is energetically more favourable than anoxic respiration (see Chapter 4), a facultative organism possessing such an alternative oxidase would be placed at a competitive advantage against a facultative organism which did not possess this oxidase. The reason for the use and synthesis of cytochrome oxidase aa_3 can also be explained on an energetic basis – a greater energetic yield is achieved through the use of cytochrome aa_3 than cytochrome *o* and this aspect is described in in Chapter 4.

<u>Conclusions</u>

In facultative denitrifying organisms, two types of cytochrome oxidase are synthesized; an inducible cytochrome aa_3 complex which develops under aerobic conditions only, and a constitutive cytochrome *o* complex which is present under both anoxic and aerobic conditions. In the electron transport pathway, electrons branch to cytochrome aa_3 from cytochrome *c* and to cytochrome *o* from ubiquinone.

APPENDIX D

MECHANISMS OF REGULATION OF ANOXIC AND AEROBIC RESPIRATION

This review serves as an extended version of the review of the mechanisms of regulation of anoxic and aerobic respiration in Part II of Chapter 4. The mechanisms described here form the basis for the biochemical model of facultative organism respiration described in Chapter 5 and as such are of critical importance to the investigation. For this reason it was considered essential to provide a resource for the reader whereby they could evaluate the research findings pertinent to specific areas of the model and make an independent assessment of the information. The research findings are presented from a historical perspective in order that the significant findings are placed in context with other work of the time.

Defining "aerobic conditions"

Aerobic denitrification in which the concentration of dissolved oxygen was not measured

Experimental artifacts responsible for aerobic denitrification

Aerobic denitrification in which the concentration of dissolved oxygen was measured

D.5 CLOSURE

reduction of nitrate, and not the oxidation of ammonia and this result was confirmed by Pasteur in the same year. However the conversion of nitrate to gaseous nitrogen forms was ascribed by all these workers to chemical and not microbiological processes.

Schoenbein (1868) working with soil denitrification was the first to attribute the loss of nitrate as gaseous products to micro–organisms and Meusel (1875) supported this proposal with a mechanism by which nitrate served as an oxidant, the atoms of nitrate being used for combustion in the same way that oxygen is used under aerobic conditions. With the wisdom of hindsight, it seems strange that it took a number of years to implicate bacteria in the *loss* of nitrate from soil, given that the *production* of nitrate in soil (i.e. nitrification) was already considered a bacterial process. Undoubtedly, one of the reasons for reluctance to accept denitrification as a bacterial process was that it was difficult to imagine that the aerobic environment required for the production of nitrate through nitrification would also be a suitable environment for the loss of nitrate through denitrification. This anomaly was partially resolved by the hypothesis that aerobic and anoxic microsites could exist side by side in soil, permitting the existence of both processes.

Although the majority of experiments concerned with the loss of nitrogen were conducted with soils, the term "denitrification", coined by Gayon and Dupetit (1882) originated from work conducted on the bacterial removal of nitrogen from sewage. In one of the first microbiologically correct series of experiments, these workers observed the biological production of nitrite, nitrous oxide and dinitrogen in nitrified sewage passed through sand columns in the absence of oxygen. Additionally, they noted that a fraction of the nitrate nitrogen was assimilated into the cells of the denitrifying bacteria.

At this point it is appropriate to indicate the dilemma facing microbiologists investigating denitrification. Whereas agronomists of the time required knowledge of the mechanisms of denitrification in order to try to limit the loss of nitrogen from soils, sanitary engineers saw the benefits of the process for the removal of nitrogen from sewage and sought to exploit it to the maximum.

In a major accomplishment in 1886, Gayon and Dupetit isolated pure cultures of two strains of denitrifying bacteria, but as with other investigators of the same period assumed that the removal of nitrate in the form of a more reduced gaseous

D.2 MECHANISMS OF REGULATION OF ANOXIC RESPIRATION

It is generally accepted that inhibition by oxygen of denitrification occurs in two ways; oxygen affects both the *synthesis* and the *activity* of denitrifying enzymes. *Synthesis* refers to the formation of the enzyme by the cell i.e. genetic expression for protein composition, incorporation of the protein subunits into the membrane, and continued production of the enzyme. *Activity* refers to the ability of the enzyme to pass electrons to the electron acceptor specific to that enzyme.

In the investigations described below into the effect of oxygen on denitrifying enzymes, workers examined two situations, (i) growing the organisms under aerobic conditions and transferring them to anoxic conditions and, (ii) growing the organisms under anoxic conditions and transferring them to aerobic conditions. This is an exceedingly fortuitous approach as far as this thesis is concerned, because, in activated sludge systems removing nitrogen and phosphorus, or nitrogen only, organisms are subjected to exactly these conditions, in moving between aerated and unaerated zones.

The manner by which *synthesis* of enzymes is affected by oxygen was usually determined in experiments in which organisms were grown under aerobic conditions under which little or no denitrifying enzymes developed, the conditions were then changed to anoxic, the subsequent development of the enzymes being monitored. The manner by which the *activity* of the denitrifying enzymes was affected by oxygen was determined by growing the organisms under anoxic conditions, such that their denitrifying enzymes were fully developed and then changing the conditions to aerobic and measuring the extent to which denitrification is affected through inactivation of the enzymes. In many facultative organisms, the denitrifying enzymes are not constitutive under aerobic conditions, and are degraded with time. Although changing the growth conditions from anoxic to aerobic provided information on the activity of the enzymes, by monitoring such systems over longer periods, workers gained information concerning the effect of oxygen on the degradation and synthesis of enzyme systems.

The approach of this review follows the general experimental approach of investigations into the effect of oxygen on denitrification. The mechanisms hypothesized as being responsible for this effect are described below and are reviewed in that order.

Organisms requiring both the absence of oxygen and the presence of nitrate for induction of nitrate reductase synthesis

- *Bacillus stearothermophilus (B. stearothermophilus)*

Downey (1966) reported that nitrate was required for growth under anoxic conditions for *B. stearothermophilus* and that although oxygen at *low* levels did not retard *induction* of the enzymes, enzyme synthesis was considerably lessened by aeration. In further work by Downey *et al.* (1969), on the same organism, grown aerobically with nitrate, low levels of nitrate reductase were reported. When the culture was switched to anoxic conditions, no immediate denitrification was exhibited but after a brief lag period ($\approx$ 1 hr) growth of the organism and production of nitrate reductase increased and denitrification was established. Nitrite reductase formation in the organism was found to be more sensitive to oxygen than nitrate reductase formation and under anoxic conditions was induced sequentially to nitrate reductase after the addition of nitrate.

- *Klebsiella aerogenes (K. aerogenes)*

Pichinoty and d'Ornano (1961 b,c) found that for *K. aerogenes*, aeration repressed production of the nitrate reducing enzyme and under anoxic conditions nitrate was required to induce its formation.

- *Paracoccus denitrificans (Pa. denitrificans)*

Lam and Nicholas (1969a) studied aerobic and anoxic respiration in *Pa. denitrificans* (formerly *Micrococcus denitrificans*) and found that cells grown aerobically and containing very little nitrate reductase, and no nitrite reductase, when transferred to anoxic conditions containing NO_3^- developed rapid nitrate reductase activity within 2–4 hours and developed rapid nitrite reductase activity within 4–6 hours. The result indicated that for *Pa. denitrificans*, nitrate reductase formation required both derepression (the absence of O_2) and specific induction (the presence of NO_3^-), but regarding the formation of nitrite reductase, no conclusions regarding the necessity for induction by NO_2^- could be made because nitrite is formed as a consequence of nitrate reduction and its presence may have induced nitrite reductase formation. To determine the requirement for NO_2^- in induction of nitrite reductase, cells of *Pa. denitrificans* grown aerobically and transferred to anoxic conditions containing NO_2^- but not NO_3^- exhibited high nitrite reductase activity but low nitrate reductase activity even after 30 hours. These results indicated that nitrate– and nitrite reductase formation require both the absence of oxygen and the presence of NO_3^- and NO_2^- respectively.

low (basal) level irrespective of the presence or absence of nitrate. Under aerobic conditions, the synthesis of nitrate reductase was repressed to its basal level. However, at concentrations of oxygen lower than saturation but at which enzyme formation was still completely repressed, the presence of nitrate induced the formation of nitrate reductase. The authors concluded that the rate of synthesis of the nitrate reducing enzyme in *E. coli* could be increased by

(i) derepression, i.e. a shift from aerobic to anoxic growth,

or

(ii) induction, i.e. the addition of nitrate to anoxic growth conditions,

or

(iii) a combination of the two situations above, i.e. a shift from aerobic conditions to anoxic conditions with nitrate present.

In each of the above transitions, synthesis of nitrate reductase was marked by two *rates, an initial rapid rate followed by a slower rate, the significance of which is described below. Concerning the levels of nitrate reducing enzyme in studies conducted with E. coli* by Showe and de Moss (1968), it was found that in highly aerated cultures, enzyme levels were 2–4% of those observed under anaerobic conditions in the absence of nitrate. Enzyme levels increased under anaerobic conditions but with nitrate present were 20 times greater than in the absence of nitrate. (Thus, the enzyme levels were between 500–1000 times greater under anoxic conditions with nitrate present, than under aerobic conditions). The researchers concluded that nitrate reductase synthesis was controlled by a redox–sensitive repressor, sensitive not only to nitrate, but also to the effective intracellular redox potential. This redox potential is a function not only of the potential oxidants (e.g. O_2, NO_3^-) and reductants (e.g. NADH) in the cell, but also of the catalytic capabilities of the cell for electron flow, (i.e. the catalytic components of the denitrification pathway preceding nitrate reductase). This aspect of redox control in denitrification is discussed further in a later section which investigates the role of redox potential of components of the denitrification pathway in the activity of the nitrogen oxide reductases. (It is interesting to note that Wimpenny and Cole (1967) in studies on *E. coli* argued for the controlling factor in nitrate reductase formation as being the redox potential of the medium). In a review of control of synthesis of nitrate reductase, Stewart (1988) suggests that the results of Showe and De Moss (1968) indicate that control by nitrate reductase and oxygen are separate, but the effect of oxygen is dominant. Ruiz–Herrera and

Organisms requiring only the absence of oxygen for induction of nitrate reductase synthesis

For the organisms described above (grown under aerobic conditions and transferred to anoxic conditions), initiation of nitrate reductase synthesis required both the absence of oxygen (derepression) and the presence of nitrate (induction). However, for other organisms (also grown under aerobic conditions and transferred to anoxic conditions) initiation of nitrate reductase synthesis required only repression by the absence of oxygen. Induction by nitrate was not required. As mentioned above, for the experiments described below, the necessity for the presence of nitrogen oxides other than nitrate in the induction of their respective nitrogen oxide reductases was not usually tested directly, but originated as a consequence of experiments with nitrate and nitrate reductase.

- *Bacillus licheniformis* (*B. licheniformis*)

Schulp and Stouthamer (1970) found that nitrate reductase formation was induced under anoxic conditions in the absence of nitrate in *B. licheniformis*, although the addition of nitrate did cause a 2½ fold increase in nitrate reductase activity.

- *Haemophilus parainfluenzae* (*H. parainfluenzae*)

Sinclair and White (1970) also found that nitrate was not necessary for the development of nitrate and nitrite reductase activities in *H. parainfluenzae* at low oxygen concentrations, although the activities of enzymes in cells grown at low oxygen concentrations with nitrate absent were about half those in cells grown at low oxygen concentrations with nitrate present.

- *Proteus mirabilis* (*P. mirabilis*)

In experiments conducted on *P. mirabilis*, de Groot and Stouthamer (1970b) found that nitrate reductase was subjected to repression and inactivation by oxygen, but when a culture of the organism growing aerobically was shifted to anoxic conditions, the formation of the enzyme was derepressed even in the absence of nitrate.

- *Pseudomonas perfectomarinus* (*Ps. perfectomarinus*)

Payne *et al.* (1971) grew *Ps.perfectomarinus* aerobically and observed the repression of denitrifying enzymes. Under conditions in which oxygen was absent, the bacteria synthesized all the denitrifying enzymes (i.e. separate nitrate, nitrite, nitric oxide and nitrous oxide reducing fractions) within 3 hours, even in the absence of the nitrogen oxides. However the authors did not state to what proportion of the

by measuring the rate of reduction of the nitrogen oxide intermediates, the effect of the intermediates on the nitrogen oxide reductases was attributed to changes in enzyme activity.

Little experimental work has been conducted on the effect at the genetic level of the nitrogen oxide intermediates on enzyme synthesis – most work has been concerned with hypothetical mechanisms for this effect and has been directed at nitrate reductase only.

In the foregoing analysis of the effect of oxygen and nitrogen oxides on the synthesis of the denitrifying enzymes, the development or lack of development of these enzymes in the presence of oxygen and absence of nitrate was referred to simply as induction or repression of formation. Because these means of regulation could not be explained using simple biochemical mechanisms, researchers were led to investigate these phenomena at the level of genetic expression of the reductases. The organism *E. coli* is one of the most studied organisms at a biochemical level particularly as far as nitrate reduction is concerned. As a result it has lent itself well to genetic studies concerned with oxygen regulation of nitrate reduction.

Several studies have concluded that control of nitrate reductase synthesis by nitrate and oxygen is at the stages of transcription and translation of gene expression. Many different mechanisms have been proposed by which such control could be executed. Ruiz–Herrera and Salas–Vargas (1976) interpreted results gained with *E. coli* shifted between aerobic and anoxic conditions as evidence that oxygen (and possibly nitrate) affect simultaneously the transcriptional and translational processes involved in nitrate reductase synthesis. In a review of nitrate respiration, Stewart (1988) concluded that biochemical pathway studies with *E. coli* such as those by Showe and de Moss (1968) indicated that nitrate and oxygen are independent regulators of nitrate reductase gene expression.

Concerning the role of nitrate, work by Stewart (1982) and Li and de Moss (1987) provided evidence that nitrate acts to induce transcription of the genes regulating nitrate reductase synthesis.

Concerning the role of oxygen in repression of nitrate reductase synthesis, Stewart (1988) states that a single regulatory protein which responds to changes in redox states induced by the presence or absence of oxygen could affect nitrate reductase

specified. Nitrate reductase is then synthesized. In the absence of oxygen, a second regulatory mechanism for enzyme synthesis (autogenous regulation) is functional and operates according to the presence and absence of nitrate.

The mechanism of control of enzyme induction by autogenous regulation was described by Pateman and Cove (1967) who identified a gene which specifies the regulatory protein described above. By itself, this protein functions as an activator for a group of genes which includes the gene for nitrate reductase synthesis. Concerning the mode of action of the regulatory protein, there are two conditions to consider; the absence of nitrate and the presence of nitrate.

(1) In the absence of nitrate, free nitrate reductase converts the protein through a change in physical form (conformation) from an activator to a repressor for a specific group of genes – one of the genes specifies nitrate reductase synthesis and with that gene repressed, the synthesis of nitrate reductase is prevented or reduced.

(2) In the presence of nitrate, nitrate reductase is bound to the nitrate molecule and cannot convert the regulatory proteins from an activator to a repressor of gene specification. Consequently, the gene is activated and nitrate reductase is synthesized. The concentration of nitrate decreases as a result of oxidation by the additional nitrate reductase and free nitrate reductase is then available to convert the regulatory protein from an activator to a repressor, i.e. a return to state (1). Thus, as intracellular nitrate concentrations increase or decrease due to corresponding changes in extracellular nitrate concentrations, nitrate reductase synthesis will be activated or repressed to accommodate these changes. These models were hypothesized to explain the regulation of nitrate reductase synthesis only although Cove and Pateman (1969) proposed that in *Aspergillus nidulans* nitrate reductase acts by this mechanism to control the rate of synthesis of nitrate reductase as well as the rate of synthesis of nitrite reductase. Due to the apparent complexity of interactions between the many intermediates and reductases, mechanisms for synthesis of the other nitrogen oxide reductases have not yet been proposed. However it would seem likely that the absence of oxygen is also responsible for derepression of synthesis of the other nitrogen oxides, since Payne (1973) found with *Ps. perfectomarina* that synthesis of all the denitrifying enzymes began simultaneously when an

similar separate anoxic conditions. The authors assumed that the rate of nitrate reduction was due to the degree of synthesis of nitrate reductase. The formation of nitrate reductase after growth under aerobic conditions varied from total repression in some bacteria to near non–repression in other bacteria. Although not stated it is implied that the authors considered that testing cell–free extracts rather than whole–cell suspension ensured that the rate of nitrate reduction was a function of the level of nitrate reductase only and not a function of nitrate reductase activity which, as will be discussed in a later section, is affected by the presence of other components of the bacterial cell which restrict the movement of nitrate to its reductase.

- In the second part of the first set of experiments conducted by Krul and Veeningen (1977), the bacterial type which exhibited the greatest nitrate reduction rate after growth at DO > 2,0 mgO/ℓ was tested for the effect of DO on nitrate reductase synthesis by growing that bacterial type at four different concentrations of DO, ranging from anoxic conditions to conditions with a DO concentration > 15 mgO/ℓ. The rate of nitrate reduction decreased as the concentration of DO increased; the nitrate reduction rate by cell–free extracts grown under anoxic conditions was five times the rate of cell–free extracts of the same organism grown under aerobic conditions at a DO concentration > 15 mgO/ℓ.

In the second set of experiments, Simpkin and Boyle (1988) conducted similar tests to those described above, the major difference being that rather than determining the activity of nitrate reductase from activated sludge organisms grown in pure culture, the activity was determined of nitrate reductase in the complete organism mass of activated sludge grown under various aerobic/anoxic conditions. As in the determinations of Krul and Veeningen (1977), the workers equated the rate of nitrate reduction in cell–free extracts of the sludge mass with the degree of synthesis of nitrate reductase. The maximum synthesis of nitrate and nitrite reductases (potential nitrate and nitrite reductase activity) was measured by examining the rates of nitrate and nitrite reduction under anoxic conditions of sludge grown under various aerobic/anoxic conditions (from < ½ hour to 4 hours anoxic period in a 6 hour cycle), the sample then held anoxic for 3 hours with nitrate present. The synthesis of nitrate and nitrite reductases under aerobic conditions (expressed nitrate and nitrite reductase activity) was measured by examining immediately after sampling the rates of nitrate and nitrite reduction under anoxic conditions with

Mechanisms for regulation of synthesis and activity of denitrifying enzymes under anoxic conditions (anoxic growth conditions)

The work above demonstrates the control exerted by oxygen on the synthesis of denitrifying enzymes when organisms grown under aerobic conditions are exposed to anoxic conditions. It is important to consider the mechanisms which control the activity of denitrification enzymes under anoxic conditions.

Under anoxic conditions and when substrate is not limiting, the denitrification rate of an organism is a function of the level of denitrifying enzyme present, the activity of the enzyme and the amount of nitrogen oxide available for reduction. It was shown that synthesis of a nitrogen oxide reductase is stimulated by its own nitrogen oxide, i.e. the presence of nitrate stimulates the synthesis of nitrate reductase. This occurs under conditions in which an organism is switched from aerobic to anoxic conditions with a nitrogen oxide present, and also under continuous anoxic conditions with a nitrogen oxide present. A factor which has not yet been considered is the effect (inhibitory or productive) of the intermediates of the process of denitrification, on the activity of enzymes which are not specific for the reduction of those intermediates, e.g. the inhibitory effect of nitrite on the activity of nitrate reductase. Because there are four denitrification intermediates including nitrate, and a reductase specific to each of these intermediates, the number of possible interactions between intermediates and reductases is considerable. Consequently, analysis of the literature is difficult and is made more complex by contradictory results between workers. The manner in which each of the reductases is affected by each of the intermediates is considered, except for cases where these interactions have not been documented.

Nitrate Reduction

Fewson and Nicholas (1961b) and Lam and Nicholas (1969b), working with *Pa. denitrificans* found that nitrate induced the formation of nitrate reductase. However they were unable to come to a conclusion regarding the effect of nitrate on the activity of nitrate reductase because of uncertainty regarding the effect of the other intermediates of denitrification formed by the reduction of nitrate. There is some disagreement between workers concerning the effect of nitrite on the activity of nitrate reductase. Kodama *et al.* (1969), De Groot and Stouthamer (1970a) and Kučera *et al.* (1983a) worked with *Ps. stutzeri*, *P. mirabilis* and *Pa. denitrificans* respectively, and found that the presence of nitrite had an inhibitory effect on the organisms ability to reduce nitrate. However, Showe and de Moss (1968) found that

et al. (1983) found that the addition of nitrous oxide to cultures of *Pa. denitrificans* strongly inhibited the reduction of added nitrite.

Nitric Oxide Reduction

Because of reluctance by many research groups to accept either nitric oxide as an intermediate in the pathway of denitrification or a specific nitric oxide reductase, little consideration had been given to the effect of the intermediates of denitrification on the reduction of nitric oxide. Payne and Riley (1969) showed that nitrate has an inhibitory effect on the activity of a nitric oxide reducing fraction from *Ps. perfectomarinus.* Payne *et al.* (1971) working on the same organism found also that nitrite inhibited nitric oxide reduction but that four to five times as much nitrite as nitrate was required under identical conditions to achieve the maximum inhibition of activity which was 60% of the total activity. Frunzke and Zumft (1986) found that *Ps. perfectomarina* grown with NO_3^- had a rate of NO reduction at least 3 times higher than when grown with N_2O, indicating that the activity of nitrogen oxide reductase is stimulated by the presence of nitrogen oxides which result in the generation of the nitrogen oxide specific to that reductase.

Nitrous Oxide Reduction

Payne *et al.* (1971) suggested that when nitric oxide is present in considerably greater excess than nitrous oxide, then nitrous oxide reduction is inhibited in an extract from *Ps. perfectomarinus.* Frunzke and Zumft (1986) found also that in *Ps. perfectomarina* nitrous oxide respiration was inhibited by nitric oxide.

A pattern in the inhibition of activity in the interactions between denitrification intermediates and reductases

A measure of caution is advised by Carlson and Ingraham (1983) in making generalizations regarding the effect of the intermediates of denitrification on each of the denitrification steps. Although comparisons of the denitrification behaviour of *Ps. stutzeri*, *Ps. aeruginosa* and *Pa. denitrificans* indicated a pattern in the *manner* in which the denitrification intermediates affect each reduction step, there are differences in the *degree* to which the denitrification intermediates affect each step. In particular, nitric and nitrous oxide reduction by *Ps. aeruginosa* was considerably more sensitive to inhibition by nitrite than either of the other two organisms tested, indicating the danger in using the behaviour of one denitrifying organism as representative of all denitrifying organisms. The results described above for the interactions between denitrification intermediates and reductases are presented in

oxide reductases was to apply the experimental results to practical situations in which denitrifying organisms in activated sludge are exposed to different concentrations of nitrate and nitrite under anoxic conditions. A problem with the experimental results is that they come from artificially manufactured situations in which the reductase to be studied is isolated from the organism and the inhibitory effects of various intermediates are tested, often at very high concentrations. This inclines one to view the denitrification pathway as a sequential step–wise process in which nitrate is completely reduced to nitrite which is then completely reduced to nitric oxide and so on to dinitrogen. However, in practice, denitrification occurs by the simultaneous reduction of nitrate, nitrite, nitric oxide and nitrous oxide, in which the inhibitory and inductive mechanisms described above ensure that none of the intermediates develop to high levels.

The simultaneous reduction of the nitrogen oxides was noted in studies with *Pa. denitrificans* by many workers, (John, 1977; Garber and Hollocher, 1981; Alefounder *et al.*, 1981, 1982, 1983; Ferguson, 1982; Boogerd *et al.*, 1981; Kučera *et al.*, 1983a; Kučera *et al.*, 1986a).

Regulation by oxygen of the activities of the denitrifying enzymes (anoxic growth conditions changed to aerobic conditions)

In the above work, mechanisms were described by which oxygen represses the synthesis of denitrifying enzymes. It is known that oxygen regulates denitrification by two mechanisms; through repression of nitrogen oxide synthesis, and through inhibition of nitrogen oxide activity.

This section examines the mechanisms involved in changes in activities of denitrifying enzymes when bacteria, grown under anoxic conditions in the presence of one of the nitrogen oxides (such that the denitrifying enzymes are developed to their maximum level and activity), are transferred to aerobic conditions. It is generally accepted that immediately upon exposure to aerobic conditions, denitrification ceases or decreases to a small fraction of the denitrification rate under anoxic conditions. Since it is improbable that such a rapid change in nitrogen oxide reduction could result from changes in the levels of denitrifying enzymes, the effect of oxygen in preventing or inhibiting denitrification is attributed to its effect on the activities of the nitrogen oxide enzymes, i.e. their electron transferring ability.

nitrite reductase.

The inactivation of the denitrifying enzymes (and nitrate reductase in particular), under aerobic conditions is hypothesized to occur by one or a combination of the following four mechanisms;

- oxygen interferes directly with the enzymes thereby preventing exchange of electrons from the reductases to the appropriate electron acceptors,
- extracellular oxygen affects the redox potential of the enzyme complexes of organism, thereby affecting the regulation of the flow of electrons to the terminal electron acceptors,
- oxygen prevents insertion of components of nitrate reductase into the cytoplasmic membrane, thereby preventing transport of electrons from the electron transferring complexes to nitrate reductase,
- oxygen affects the permeability of the cytoplasmic membrane, thereby regulating the movement of nitrate to nitrate reductase, which in turn prevents electron transport from nitrate reductase to nitrate.

Inactivation of the nitrogen oxide reductases through direct interference by oxygen
An obvious mechanism by which oxygen could inactivate the nitrogen oxide reductases is through direct attachment to, or reaction with the reductases, in a manner which prevents the transfer of electrons to the nitrogen oxide electron acceptors. However, in research into this effect, no such mechanism has been found and as proof that oxygen does not act in this manner, workers have considered it sufficient to demonstrate that oxygen inhibits nitrogen oxidase reduction in a manner other than through direct interference with the reductases.

Payne (1973) suggested that inactivation of nitrogen oxide reductase results from oxygen acting as an electron acceptor rather than from an effect of oxygen *per se.*

Stouthamer (1988) excluded the possibility that inactivation occurs through the direct interference by oxygen, on the basis of the following experimental observations:

- Alefounder *et al.* (1981) and Kučera *et al.* (1981) working independently, with *Pa. denitrificans*, used an artificial electron acceptor, ferricyanide, (it accepts electrons from cytochrome *c*, Boogerd *et al.*, 1981, van Verseveld *et al.*, 1981) to

Inhibition by oxygen of electron flow to the nitrogen oxide reductases as a result of changes in the redox potential of components of the electron transport pathway

In early work on the effect of oxygen on nitrate reductase, considerable variation was reported in the degree to which oxygen affected the activity of the nitrogen oxide reductases. However, irrespective of the degree to which oxygen affected this activity, workers such as Stickland (1931) concluded that the manner by which oxygen affects the flow of electrons to nitrate via nitrate reductase, is by redirecting the electrons to oxygen via cytochrome oxidase, but because of a lack of understanding concerning the complexes comprising the aerobic and anoxic pathways they did not propose specific mechanisms as to how this could occur. Workers such as Fewson and Nicholas (1961b) noted a loss of nitrate reductase activity in *Ps. aeruginosa* under aerobic conditions and attributed it to the diverting of electrons away from the denitrifying pathway and nitrate reduction, and to the aerobic pathway and the reduction of oxygen.

In determining the control exerted by oxygen on the denitrifying enzymes of *K. aerogenes*, van't Riet *et al.* (1968) subjected a culture of the organisms to a shift from anoxic conditions to aerobic conditions and noted an immediate cessation of nitrite production from nitrate reduction. They suggested that oxygen inactivates nitrate reductase and represses its further formation but did not propose a mechanism to explain either inactivation or repression. However, further work by van't Riet *et al.* (1972) with *K. aerogenes* implicated a component or components of the respiratory chain as being responsible for the withdrawal of electrons from denitrifying enzymes under aerobic conditions. This implication arose from experiments in which purified nitrate reductase from *K. aerogenes* was not inactivated by oxygen whereas cell free, nitrate reducing extracts (which besides the nitrate reducing fraction also contained other electron transferring components of the respiratory pathway still associated with the cytoplasmic membrane) from the same organism were inactivated.

In similar experiments, Pichinoty and d'Ornano (1961c) working with the same organism also did not observe inactivation of purified nitrate reductase by oxygen.
The inactivation of enzymes under aerobic conditions was noted by de Groot and Stouthamer (1970a) with *P. mirabilis*. The authors assessed nitrate reductase activity by examining (i) the rate of nitrate reduction of cell-free extracts of the culture under anoxic conditions and (ii) the rate of nitrite accumulation of whole-cell suspensions of the culture under anoxic conditions. After shift of the

branching point to nitrate reductase at cytochrome *b* and the branching point to nitrite reductase and the cytochrome oxidases at cytochrome *c* (see Fig C.3). Stouthamer *et al.* (1980) included nitrous oxide reductase accepting electrons from the cytrochrome cc_1 complex (see Fig C.4). Later schemes by Vignais *et al.* (1982), Whatley (1981) and Knowles (1982) established the electron pathway branching to nitrate reduction and to terminal oxidase at the bc_1 complex or from cytochrome *b* and cytochrome *c* respectively (see Fig C.5). In a more recent scheme, Ferguson (1982) proposed that nitrate reductase gains electrons from ubiquinone rather than from the cytochrome *bc* complex, cytochrome oxidase gains electrons from cytochrome *c* (see Fig C.6) and alternative cytochrome oxidase (cytochrome *o*) gains electrons from ubiquinone. It is important in the following review of the involvement of different components of the electron transport pathway in the control of electron flow, that the state of knowledge of the components of the pathway at the time of the research be taken into account. It may be necessary to refer to the electron transport scheme applicable at the time to understand the research.

- *Redox control of electron flow to nitrate reductase exerted by cytochrome b:*

Prior to the scheme of Ferguson (1982), other schemes for the denitrification pathway (Knook *et al.*, 1973; John and Whatley, 1975; Stouthamer, 1980; Knowles, 1982) considered the branch point to nitrate reductase to be situated at cytochrome *b*. In such schemes cytochrome *b* could have a controlling function over the flow of electrons to nitrate reductase. Alefounder *et al.* (1983) considers this unlikely given an acceptance of the scheme of Ferguson (1982) (see Fig C.6), in which cytochrome *b* occurs in the respiratory chain after the branch point to nitrate reductase. It would then be difficult to envisage that nitrate reductase could be sensitive to the redox state of a component on the oxidizing side of the branch point to nitrate reductase. Also, nitrate reduction is inhibited both when electron flow is through cytochrome *b* (i.e. in the presence of added nitrous oxide which diverts electrons away from nitrate reductase to nitrous oxide reductase) (Kučera *et al.*, 1983a; Alefounder *et al.*, 1983).

- *Redox control of electron flow to nitrate reductase exerted by cytochrome cd:*

Experiments conducted by Kučera *et al.* (1981) with ferricyanide and hydroxylamine led the authors to suggest that the redox state of cytochrome *cd* (nitrite reductase) which has cytochrome oxidase activity in the presence of oxygen and gains electrons from cytochrome *c* might play an important role in the control of nitrate reduction

electron flow to cytochrome oxidase aa_3, would permit a low rate of electron flow to nitrate reductase. Conversely, a high extent of reduction of ubiquinone resulting from a low rate of electron flow to cytochrome oxidase aa_3 would permit a high rate of electron flow to nitrate reductase. The extent of reduction of ubiquinone could then directly or indirectly operate the switch for controlling nitrate reduction by regulating the movement of electrons or nitrate to nitrate reductase.

The scheme above has considerable appeal in the light of the respiratory electron pathway proposed by Ferguson (1982) in which ubiquinone is the last common component in electron flow to nitrate reductase and terminal oxidase.

Although no direct experimental evidence has been obtained to implicate ubiquinone as having a controlling function, Alefounder *et al.* (1983) found indirect evidence to suggest that following a transition from aerobic to anoxic conditions in which nitrate reduction was initiated, the extent of reduction of ubiquinone was increased. They concluded that relatively small changes in the oxidation state of ubiquinone could control the extent of nitrate reduction by control of electron flow to nitrate.

Because of the difficulty in measuring the redox state of intracellular components of the respiratory chain, there is little experimental evidence to support the hypothesis, but conversely little experimental evidence exists to disprove it.

Prevention by oxygen of the insertion of the nitrate reductase subunits into the cytoplasmic membrane

Stewart (1988), in reviewing the regulation of nitrate reductase activity by oxygen in *E. coli*, considers that in a shift from anoxic to aerobic conditions in which nitrate reductase cannot function in electron transport, nitrate reductase (and other anoxic respiratory enzymes) cannot be incorporated into the membrane. This hypothesis is based on the following three findings.

- De Groot and Stouthamer (1970b), showed that the formation of reductases is repressed under conditions when electron transfer to the reductases cannot occur. For example, under aerobic conditions in which electron flow is to terminal oxidase only, the formation of nitrate reductase would be repressed.

- MacGregor (1976) found that a function of the cytochrome *b* (γ) subunit of nitrate reductase is to associate the nitrate reductase complex with the

the organisms were grown under anoxic conditions (such that all denitrifying components of the respiratory chain were present); the effect of oxygen on nitrate movement across the membrane was then investigated by gradually introducing oxygen into the anoxic environment and monitoring changes in reduction of the nitrogen oxides with time. In another approach, organisms were grown under anoxic conditions, and rapidly transferred to aerobic conditions under which denitrification did not proceed, or proceeded at a considerably reduced rate. The factors required to initiate denitrification (or nitrate reduction) under aerobic conditions by effecting changes in the cytoplasmic membrane were then determined.

- *Intact cells versus inside–out membrane vescicles*

John (1977) investigated the effect of oxygen on the reduction of nitrate by whole cells and with membrane vescicles. In the first test, intact cells of anoxically grown *Pa. denitrificans* having nitrate reductase and cytochrome oxidase activities were placed under aerobic conditions with nitrate present. Nitrate reduction commenced only when all the oxygen present had been consumed. The inhibitory effect of oxygen on nitrate reduction was shown repeatedly by reintroducing oxygen in the presence of nitrate such that nitrate reduction ceased. In the second experiment, inside out membrane vescicles of anoxically grown *Pa. denitrificans* with nitrate reductase and cytochrome oxidase activities (in which access by nitrate to nitrate reductase was not prevented by the cytoplasmic membrane) were subjected to the same aerobic conditions as in the first experiment. The membrane vescicles reduced oxygen and nitrate simultaneously but when the oxygen had been consumed there was little increase in the rate of nitrate reduction. Thus, in intact cells, electrons are used preferentially by oxygen, but in membrane vescicles, electrons were distributed between both nitrate reductase and cytochrome oxidase indicating that under aerobic conditions the cytoplasmic membrane plays an important role in the regulation of nitrate reduction.

- *Chemical permeabilization of cytoplasmic membrane*

Alefounder and Ferguson (1980) also implicated the cytoplasmic membrane in the inhibition of nitrate reduction. Spheroplasts of *Pa. denitrificans* treated with Triton X–100 (a chemical which permeabilizes the cytoplasmic membrane allowing the movement of nitrate to nitrate reductase), reduced nitrate and oxygen simultaneously under aerobic conditions. The increased flow of electrons to nitrate reductase due to the availability of nitrate, led to a decrease in the flow of electrons to the oxidases, the rate of oxygen reduction being only half the rate measured in

act in a manner similar to reduced ubiquinone and donate electrons to nitrate reductase, cytochrome *o* and the cytochrome bc_1 complex). It was found that the addition of durohydroquinone did not result in nitrate reduction under aerobic conditions and the authors concluded that a good case could be made for control being exerted on nitrate movement across the cytoplasm rather than on preferential flow of electrons to oxygen.

Although the majority of work with this technique was conducted on the bacterium *Pa. denitrificans*, other workers investigated the effect of oxygen on the movement of nitrate in other organisms using different techniques.

- *Use of a transmembrane nitrate carrier*

Noji and Taniguchi (1987) studied the control of nitrate reduction in *E. coli* and concluded that control is primarily due to restriction of nitrate movement to its reductase on the cytoplasmic side of the membrane. Under aerobic conditions nitrate reduction was absent but under aerobic conditions in the presence of benzyl or heptyl viologen radicals (reported to be trans-membrane nitrate carriers, or ionopores) nitrate reduction increased. The workers suggested that oxygen may interact at a specific nitrate transport site in the electron transport chain especially if nitrate and nitrite movement across the membrane occurs by means of a nitrate/nitrite antiport system as suggested by Jones *et al.* (1980) and Boogerd *et al.* (1983a). However they also presented an alternative explanation for the impermeability of the membrane to nitrate under aerobic conditions; the transport mechanism for nitrate across the membrane is stimulated by the absence of oxygen rather than inhibited by the presence of oxygen.

- *Intact cells versus cell extracts*

Hernandez and Rowe (1987) studied the effect of oxygen at various concentrations on nitrate respiration in *Ps. aeruginosa* and found the inhibitory effect to be a maximum at 0,2% of oxygen saturation. Their results indicated the cause of inhibition to be at the level of nitrate uptake since inhibition was observed in whole cell preparations but not in cell extracts in which the cytoplasmic membrane was destroyed. An important finding in this work was that nitrite uptake by whole cells was not inhibited by the low concentrations of oxygen which inhibited nitrate reduction, and at higher concentrations of oxygen, nitrite reduction by whole cells was only partially inhibited. These results can be explained by (i) the periplasmic orientation for nitrite reductase (thereby eliminating the necessity for extracellular

denitrifying enzymes, since reversal of the inactivation may require anoxic conditions.

In conclusion, the importance of the orientation of nitrate reductase with the cytoplasmic membrane, with respect to the effect of oxygen on nitrate reduction is demonstrated in an interesting piece of work conducted by Bell *et al.* (1990) on the organism *Thiosphaera pantotropha.* In this organism a periplasmic nitrate reductase develops under both aerobic and anoxic conditions while the usual cytoplasmically oriented nitrate reductase develops under anoxic conditions only (as is the case for most facultative denitrifiers). The organism has the unusual ability to reduce nitrate at high rates under aerobic conditions and this ability is attributed to the periplasmically oriented nitrate reductase developed under aerobic conditions, an orientation which allows nitrate reduction to occur without the necessity for nitrate to cross the cytoplasmic membrane to be reduced. For all other organisms studied in this review, nitrate reductase is situated on the cytoplasmic side of the membrane and under aerobic conditions nitrate reduction does not occur, or occurs only at very low rates. Under anoxic conditions in the presence of nitrate, organisms with a cytoplasmic placement of nitrate reductase reduce nitrate at high rates. Thus, it appears that oxygen affects the permeability of the cytoplasmic membrane; under aerobic conditions the cytoplasmic membrane restricts the movement of nitrate to the cytoplasmic side, thereby inhibiting nitrate reduction. This conclusion is supported by the finding that nitrite reduction is inhibited only partially by oxygen as a consequence of the periplasmic orientation of nitrite reductase.

D.3 MECHANISMS OF REGULATION OF AEROBIC RESPIRATION

Introduction

In Appendix C the characteristics and the function of terminal cytochrome oxidase were outlined. It was shown that two types of cytochrome oxidase are synthesized by denitrifying, facultative organisms. These are the enzyme complexes, cytochrome aa_3 and cytochrome *o*; the former is an inducible enzyme synthesized by organisms under aerobic conditions only and the latter is a constitutive enzyme present under both aerobic and anoxic conditions.

It was shown above, that enzymes can be regulated in two ways; by regulation of the synthesis of the enzyme, and by regulation of the activity of the enzyme. In the case of denitrifying enzymes these two aspects are affected by oxygen, nitrate, and the intermediates of the process of denitrification (NO_2^-, NO and N_2O). Since the

concentration from nitrate reduction under aerobic conditions was responsible for a decrease in oxidase activity. Investigation of this mechanism forms the basis of the following sections.

The mechanism of inhibition by nitrite (NO_2^-) of oxygen utilization

The aspect of nitrite inhibition of oxygen reduction, was investigated by Kučera and Dadak (1983) with anoxically grown cells of *Pa. denitrificans* subjected to aerobic conditions in the presence of an uncoupler, a chemical which reduces the transmembrane potential of the cytoplasmic membrane, allowing access of nitrite to the cytoplasmic side. As a means of determining the distribution of electrons between cytochrome oxidase and nitrite reductase, the redox potentials of the two enzyme complexes were measured. In the presence of the uncoupler and a high concentration of nitrite when the membrane potential was lowered, electron flow to cytochrome oxidase decreased and electron flow to nitrite reductase increased as a result of nitrite gaining access to the inhibitory site of cytochrome oxidase. The manner by which nitrite interfered with cytochrome oxidase was not specified by the authors.

A problem concerning the above experiment is that it is not clear as to whether electron flow to cytochrome oxidase was inhibited as a result of the direct interaction of nitrite with cytochrome oxidase at a specific inhibitory site, or whether the initiation of nitrite reduction led to a withdrawal of electrons from the electron transport branch of cytochrome oxidase.

A mechanism was suggested by Rowe *et al.* (1979) by which nitrite could inhibit oxidase activity in *Ps. aeruginosa.* Nitrite apparently exerted its effect by oxidizing the ferrous (Fe^{2+}) ion of cytochrome oxidase to the ferric (Fe^{3+}) form, thereby preventing the transfer of electrons to oxygen. Also, Yang (1985) investigated the inhibitory effect of nitrite on cytochrome oxidase in *Ps. aeruginosa.*

The addition of nitrite at concentrations of 1–5 M inhibited oxidase activity but interestingly, nitrate, at concentrations much higher than that of nitrite had little or no inhibitory effect. No explanation was offered for the lack of inhibition by nitrate, but it is likely that the explanation given above for the prevention of nitrate reduction under aerobic conditions applies; the presence of oxygen affects the permeability of the cytoplasmic membrane, thereby preventing the movement of nitrate to nitrate reductase situated on the cytoplasmic side, and preventing the

responsible for inhibition of cytochrome oxidase after it was shown that cytochrome oxidase could be inhibited by the addition of nitric oxide but not by the addition of nitrous oxide.

Support for the finding that inhibition of terminal oxidase activity is due not to nitrite was presented by the Ferguson group. Parsonage *et al.* (1985) concluded from work on *Pa. denitrificans* with a permeabilizing agent and an uncoupler that the inhibitory species is a reaction product or an intermediate of the nitrite reductase reaction and is due not to nitrite alone. This conclusion arose as a corollary to the following findings;

- In cultures treated with the permeabilizing agent Triton X–100 or with an uncoupler which allows the movement of ions across the cytoplasmic membrane, very low concentrations of nitrite strongly inhibited electron flow to oxygen, whereas in cultures not treated with either of these two chemicals, similar low concentrations of nitrite did not inhibit electron flow to oxygen.

- With inside–out vesicles (in which periplasmically situated nitrite reductase was on the inside of the vesicle, reducing access of nitrite to it) treated with Triton X–100 or an uncoupler, low concentrations of nitrite did not inhibit electron flow to oxygen.

- In aerobically grown cultures (in which nitrite reductase was absent) treated with Triton X–100 or an uncoupler, low concentrations of nitrite did not inhibit electron flow to oxygen.

These results indicated that it was not nitrite *per se*, but a product of the catalytic activity of nitrite reductase that was responsible for the inhibition of electron flow to oxygen.

The Ferguson group considered two possible products or intermediates of nitrite reduction, nitric oxide (NO) and nitroxyl anion (NO^-) which could inhibit electron transport to oxygen by reacting with haem (Fe) centres. Nitric oxide was rejected as an inhibitory species on the grounds that, although nitric oxide is known to have a high affinity for oxygen, no oxygen consumption was measured during periods of inhibition as a result of the reaction of nitric oxide with oxygen. Because inhibition required either collapse of proton motive force by an uncoupler or permeabilization

rate of oxygen was not inhibited by the addition of nitrate or nitrite, and was inhibited only slightly by the addition of nitric oxide. From these experiments it was concluded that inhibition resulting from the addition of nitrate, nitrite or nitric oxide, was induced as a consequence of the formation of nitric oxide, the added nitrate and nitrite being reduced in turn to nitric oxide. The reason for the greater degree of inhibition measured for organisms subjected to 24 hour aerobic pretreatment than for organisms without pretreatment, was a result of the effect of aerobic conditions on the synthesis of the nitrogen oxide reductases. Lowered synthesis of nitric oxide reductase would result in a decreased rate of removal of nitric oxide of organisms subjected to aerobic pretreatment. This is supported by findings described earlier which indicated that aerobic conditions affect the activity of nitric oxide reductase to a greater degree than they do nitrite and nitrate reductase, leading to an accumulation of nitric oxide during subsequent denitrification of nitrate and nitrite.

A similarity was noted between the aerobic pretreatment/anoxic growth with nitrate conditions to which the inhibited *Alcaligenes* sp. was subjected, and the unaerated/aerated cycles to which activated sludge is subjected in N and N & P removal configurations. This similarity provided the basis for the hypothesis which explains the proliferation of filaments in aerated/unaerated systems. A fundamental aspect of the hypothesis is the inhibition of oxygen utilization of organisms in the sludge by nitric oxide, and in order that the hypothesis has credibility, it is essential that the mechanism of inhibition of oxygen utilization by nitric oxide is well established.

The remainder of this section is devoted to verification that nitric oxide is inhibitory to utilization of substrate under aerobic conditions.

In the work of the Kučera group (i.e. Kučera *et al.*, 1986b), the inhibition of oxidase activity was ascribed initially to the interaction of nitrite with cytochrome oxidase, and then to the interaction of nitric oxide with cytochrome oxidase.

Work by Kučera *et al.* (1987b) on cultures of *Pa. denitrificans* which followed the work of Kučera *et al.* (1986b) attempted to conclusively identify the inhibitory agent. The researchers added an uncoupler to an aerobic suspension of anoxically grown (with nitrite) *Pa. denitrificans* in order to induce aerobic denitrification of NO_2^-. From previous work it was suspected that the inhibitory species produced

nitric oxide was that, because of its extremely reactive nature, it would be present only in very small and unmeasurable quantities, if at all, and certainly not in quantities sufficient to inhibit cytochrome oxidase (Parsonage *et al.*, 1985). The Ferguson group opted for nitric oxide as the inhibitory species only after Carr and Ferguson (1990b) showed that *Pa. denitrificans* possessed a separate nitric oxide reductase that was active in the presence of oxygen and maintained the steady-stage dissolved nitric oxide concentration sufficiently low that reaction with oxygen was insignificant but sufficiently high that reaction with the active site of cytochrome oxidase would inhibit oxygen reduction.

It is interesting that in the work described above which concluded that nitric oxide inhibits oxidase activity, no mechanism was described for the inhibitory action of nitric oxide. However in some related work on the inhibition of respiratory processes by nitrite and nitric oxide with a photodenitrifier and a pathogenic organism, mechanisms were proposed and may have application to inhibition of respiration in facultative denitrifying organisms. Satoh (1984) working with the photodenitrifier *Rhodopseudomonas sphaeroides* forma sp. *denitrificans* proposed that nitric oxide inhibited electron transfer through the cytochrome bc_1 complex. However, since the reduction of nitric oxide also requires the transfer of electrons through the cytochrome bc_1 complex it is improbable that nitric oxide would act such that its own reduction would be inhibited.

Reddy *et al.* (1983) investigated nitrite inhibition of *Clostridium botulinum*. Nitrite, added to cells of the organism, formed nitric oxide which reacted with iron–sulphur enzymes of the respiratory electron transferring complexes forming iron–nitric oxide complexes thereby inhibiting electron transfer.

Inhibition of electron transfer (apparently by the same mechanism) also occurred after the addition of nitric oxide, Salerno *et al.* (1976), providing further evidence that the inhibitory species is nitric oxide and not nitrite.

Because early work on the inhibition of oxidase activity considered that nitrite alone was responsible for the inhibition of oxidase activity, the function of the cytoplasmic membrane was accorded considerable significance due to the orientations of nitrate reductase on the cytoplasmic side and nitrite reductase on the periplasmic side of the membrane. The only source of nitrite which could interact with cytochrome oxidase, which is situated on the cytoplasmic side, is that which arises from the

resulted from inhibition by nitrite of cytochrome *o*, the only oxidase synthesized under limited aeration conditions. Further, cultures of the organism grown aerobically such that cytochrome aa_3 was synthesized, were not inhibited by nitrite, even at excessive concentrations indicating that nitrite does not inhibit cytochrome aa_3. The second factor which implicates cytochrome *o* as the cytochrome subject to inhibition is a difference between cytochrome *o* and cytochrome aa_3 in chemical composition. Cytochrome *o* contains haem–iron (i.e. a complex in which the iron atom is bound to an organic complex) (Poole, 1982); the reactive centre of the enzyme is associated with a sulphur protein (Ferguson, 1982) referred to as an FeS complex. Cytochrome aa_3 contains four metal atoms, two iron atoms and two copper atoms (Poole, 1982), referred to as an FeCu complex. From the literature it is unclear as to whether the Fe atoms are associated with sulphur proteins. In Chapter 5 it is assumed that the FeCu complex has sulphur associated with the Fe and Cu atoms, i.e. cytochrome aa_3 contains FeS and CuS centres. It has been shown that nitric oxide exerts its inhibitory effect through its interaction with iron–sulphur (FeS) complexes to form iron–nitric oxide complexes which cannot then transfer electrons. It can be concluded that nitric oxide interacts with the FeS centres of cytochrome *o* and cytochrome aa_3. There is no evidence to suggest that nitric oxide also interacts with the CuS centres of cytochrome aa_3.

<u>Explanation for the development of the alternative oxidase cytochrome *o*</u>

As discussed earlier and from an examination of the proton pumping sites passed by electrons moving to either of the oxidases as indicated in Fig C.9, it is more beneficial energetically for oxygen to serve as electron acceptor at cytochrome aa_3 than at cytochrome *o*. Considering that the only other apparent difference between the oxidases is that cytochrome *o* is synthesized under anoxic conditions under in which oxygen is not present, the advantage afforded an organism possessing cytochrome *o* is not immediately apparent.

The advantage of a constitutive enzyme is that under conditions which change between aerobic and anoxic the organism can make immediate use of the electron acceptor specific to that condition without firstly synthesizing the enzyme. The disadvantage of constitutive enzymes is that if the conditions for which that enzyme is specific, occur infrequently, considerable energy is expended in the maintenance of the enzyme without any energetic return from its use.

Using this analysis of the advantages and disadvantages of constitutive and

nitrite reduction are noted. The review then outlines the variation in results between organisms as well as the variation in results between researchers working with the same organism.

Defining "aerobic conditions"

In reviewing the results of the experiments investigating aerobic denitrification it is difficult to determine whether the conditions under which denitrification is reported are truly aerobic. One reason is that it is difficult to *define* the concentration of oxygen that constitutes an aerobic environment and the degree of exclusion of oxygen that constitutes an anaerobic or anoxic environment. Another reason is that it is difficult to *measure* very low concentrations of oxygen and especially to be sure that anoxic sites do not exist in situations in which low concentrations of oxygen are measured in the liquid or the medium surrounding the cultures.

Regarding the definition of aerobic and anoxic environments, many early workers in the field of denitrification regarded the total exclusion of oxygen as a prerequisite for denitrification, but other workers were not so rigorous in their requirements for anaerobiosis.

Regarding the measurement of concentration of oxygen, in some experiments the concentration of oxygen was not measured either in the headspace above the culture or in the culture medium but conditions were assumed to be aerobic after sparging the culture with air or after vigorous shaking or stirring. In the first reports of aerobic denitrification the workers did not measure the concentration of dissolved oxygen in the liquid or medium, but measured the partial pressure of oxygen in the headspace above the liquid or medium and considered that physically vigorous aeration would ensure aerobic conditions in the liquid or medium, at most to the degree of the partial pressure of oxygen in the headspace. This rather qualitative approach to ensuring an aerobic environment was compounded by the technical inability in early experiments to measure the concentration of dissolved oxygen. In later reports of aerobic denitrification, although the concentration of dissolved oxygen in the bulk liquid was measured it was not certain that the concentration of dissolved oxygen measured in the bulk liquid was the same as that on the inside of a microbiological culture in which anoxic microsites could exist. Because of the lack of quantitative data in these experiments the means of comparing the effect of oxygen on denitrification between organisms is to summarize the important aspects of the experiments described by the authors. In most of these experiments, the

denitrification such as these, in which the level of dissolved oxygen was not measured are always subject to a degree of scepticism because it is uncertain as to whether the transfer of oxygen from the gaseous phase to the liquid phase is slower than the rate of oxygen utilization in the liquid phase; if this is the case, then the liquid phase is essentially anoxic.

Experimental artifacts responsible for aerobic denitrification

Collins (1955) investigated the effectiveness of aeration in laboratory grown cultures by using various flask shapes for the growth of denitrifying organisms and illustrated the effect of that factor on aeration. From that work it was concluded that many reports of aerobic denitrification may have been due to inadequate aeration, reflecting the existence of anaerobic micro–environments. In demonstrating that the degree of nitrate reduction under aerobic conditions varied with the manner of aeration and the type of organism used, Verhoeven (1956) also found that experimental artifacts could influence results. It was concluded that reports of aerobic denitrification in which the concentration of dissolved oxygen was not measured [such as that by Meiklejohn (1940) with *Pseudomonas* sp. species] could be criticized with some justification.

Aerobic denitrification in which the concentration of dissolved oxygen was measured

One of the first investigations to measure the level of dissolved oxygen in studies into aerobic denitrification was conducted by Skerman *et al.* (1951) with a culture of *Pseudomonas* sp. Concentrations of nitrate and nitrite were also measured and it was concluded that oxygen and nitrate were used simultaneously. (Disappearance of nitrate was not due to consumption for assimilatory purposes because workers of the time were aware of the assimilatory nitrogen requirement of denitrifying organisms and accommodated this by supplying sufficient ammonium in the growth medium).

Because of the influence on aerobic denitrification of the artifacts described above, and in order to ensure that reported examples of aerobic denitrification were credible, almost all subsequent investigations in this field (and those reviewed below) measured concentrations of oxygen, nitrate and nitrite in the medium.

Variability between organisms in their sensitivity to oxygen is apparent in comparing the above results in which it was concluded that organisms reduce nitrate and nitrite under aerobic conditions, with those of John (1977) with *Pa. denitrificans* and *E. coli* in which the presence of oxygen prevented the

(i.e. ≈ 0,1 mgO/ℓ). As discussed earlier, Hernandez and Rowe (1988) found that 11 out of 12 widely divergent denitrifying bacterial species ceased nitrate reduction upon exposure to oxygen and only recommenced after all the oxygen had been consumed. The exception, *Enterobacter aerogenes*, continued to reduce nitrate at a concentration close to 10% of the dissolved oxygen saturation concentration. Such diverse findings illustrate why controversy has dogged research in the field of aerobic denitrification.

The controversy as to whether aerobic denitrification is a general mechanism among denitrifying organisms was further compounded by the work of Robertson and Kuenen (1983, 1984a,b, 1990) who isolated the organism *Thiosphaera pantotropha* from a nitrogen removal waste–water treatment system. The organism has the ability to reduce nitrate and oxygen simultaneously and to nitrify heterotrophically. Reduction of nitrate under aerobic conditions is attributed to a constitutive nitrite reducing enzyme system as opposed to the inducible nitrate reducing enzyme system of other denitrifiers. The authors did not propose these characteristics as being general among denitrifiers but the results do highlight the considerable diversity and variability amongst denitrifying organisms. They suggest that differences in the reported denitrifying ability of the same organism such as with *Pa. denitrificans* and *Ps. aeruginosa* may result from research groups experimenting with different strains of the same organism.

Aerobic denitrification was noted by Krul (1976) in activated sludge, and in that work, which was discussed earlier it was noted that the rate of nitrate reduction in activated sludge under aerobic conditions was about 20% of the rate in the absence of oxygen. (The concentration of dissolved oxygen under conditions described as aerobic was not stated by the authors). This reference appears to be the only work which links aerobic denitrification of nitrate and nitrite to the effect of nitric oxide in inhibiting aerobic respiration.

D.5 CLOSURE

The objective in reviewing reports of aerobic denitrification was to establish whether the hypothesized mechanism for aerobic denitrification has support. The review established that there is a great diversity among organisms in denitrification behaviour under aerobic conditions. Some organisms ceased denitrifying at very low concentrations of oxygen whilst others continued to denitrify at concentrations of oxygen near air–saturation. For organisms which denitrified under aerobic

APPENDIX E

NITRIFICATION

Appendix E reviews the biochemical mechanisms associated with oxidation of ammonium/ammonia (NH_4^+/NH_3) to nitrite (NO_2^-) by *Nitrosomonas* and the sequential oxidation of nitrite to nitrate (NO_3^-) by *Nitrobacter*. The effect of unfavourable conditions on these organisms is analysed, in particular the conditions which give rise to high concentrations of nitrite in mixed cultures of the two organisms.

E.1 INTRODUCTION

The process of nitrification has been reviewed for two reasons: Firstly, in nitrogen (N) and nutrient (N & P) removal biological wastewater treatment systems in which nitrification is a major and essential process, bulking is a common problem, and secondly, the process of denitrification which has been implicated in bulking (see Chapter 6) requires the endproducts of nitrification (nitrate and nitrite) as reactants.

In the early 1800s Sir Humphrey Davey suggested that the presence of nitrate in soils resulted from the oxidation of ammonia. However the oxidation process was considered to be chemical until Pasteur proposed that it was microbiologically mediated. This proposal gained considerable acceptance from the experiments of Schloesing and Müntz (1877) which demonstrated that no further nitrification was measured in sewage effluent subjected to processes which destroyed the microorganisms, i.e. heat treatment or the addition of chloroform.

Winogradsky (1890) firmly established the biological nature of nitrification after isolating and describing different bacteria that oxidize ammonium (NH_4^+) to nitrite (NO_2^-), and then nitrite to nitrate (NO_3^-), naming the bacteria *Nitrosomonas* and *Nitrobacter* respectively. The concept of chemoautotrophic growth[1] developed from his realization that the organisms did not require reduced carbon compounds but could utilize carbon dioxide as their source of carbon and could obtain energy from the oxidation of ammonium.

Little more significant work was conducted in the field until the 1960s when improvements in technology allowed the extraction and characterization of the enzymes responsible for production of the nitrite and nitrate, the endproducts of the two stage oxidation process.

E.2 MECHANISMS OF NITRIFICATION

The two steps of the oxidation process for the conversion of ammonium to nitrate are conventionally written as shown in Eqs E.1 and E.2.

$$NH_4^+ + \frac{3}{2}\,O_2 \rightarrow NO_2^- + 2H^+ + H_2O \qquad \textit{Nitrosomonas} \qquad \text{(E.1)}$$

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[1] Growth with inorganic material as the sources of carbon and energy:– for a description of organism categorization see Chapter 4.

$$2H^+ + 2e^- + O_2 + NH_3 \rightarrow NH_2OH + H_2O \text{ [2]} \qquad \text{(E.3)}$$

Ammonia monooxygenase (AMO)

$$H_2O + NH_2OH \rightarrow 4e^- + 4H^+ + HNO_2 \text{ [3]} \qquad \text{(E.4)}$$

Hydroxylamine oxidoreductase (HAO)

$$2H^+ + \tfrac{1}{2} O_2 + 2e^- \rightarrow H_2O \qquad \text{(E.5)}$$

Cytochrome oxidase

(iii) The source of oxygen for oxidation: It is conventional to consider molecular oxygen (O_2) as this source for both steps in the oxidation of ammonia to nitrite. In the first oxidation step, molecular oxygen serves as the source of the oxygen atom for hydroxylamine (Eq E.3). In the second oxidation step, the oxygen atom of water serves as the second oxygen atom in the formation of nitrite, shown in the associated nitrous acid (HNO_2) and not the dissociated ($NO_2^- + H^+$) nitrite ion form (Eq E.4). Thus in the two–step oxidation of ammonia to nitrite, molecular oxygen has a dual role; (i) to donate an oxygen atom in the first oxidation step in the formation of hydroxylamine and, (ii) as an electron acceptor for two of the electrons produced in the second oxidation step (the other two electrons are returned to AMO for the formation of hydroxylamine). Evidence in support of these mechanisms is as follows: Rees and Nason (1966) using isotopically labelled oxygen atoms in molecular oxygen and in water determined that one, but not both of the oxygen atoms in nitrite is derived from molecular oxygen, the other presumably derived from water; subsequently, Dua *et al.* (1979), and Hollocher *et al.* (1981), also using labelled isotopes, established that the oxidation step of ammonia to hydroxylamine involved incorporation of oxygen from molecular oxygen. Based on these results, it was concluded that oxygen incorporated in the oxidation step of hydroxylamine originates from water, a conclusion verified by Andersson and Hooper (1983) using isotopically labelled oxygen and nitrogen.

(iv) The enzyme complexes associated with the two oxidation steps to nitrite: Two groups of enzymes separately catalyze the reactions; an ammonia oxygenase catalyzing the oxidation of ammonia to hydroxylamine and an hydroxylamine

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[2] Ammonia is shown in its dissociated (NH_3) and not its associated (NH_4^+) form for the reasons given above.

[3] In practice, a quantity of N_2O appears as an endproduct; the conditions under which this occurs are discussed at a later stage.

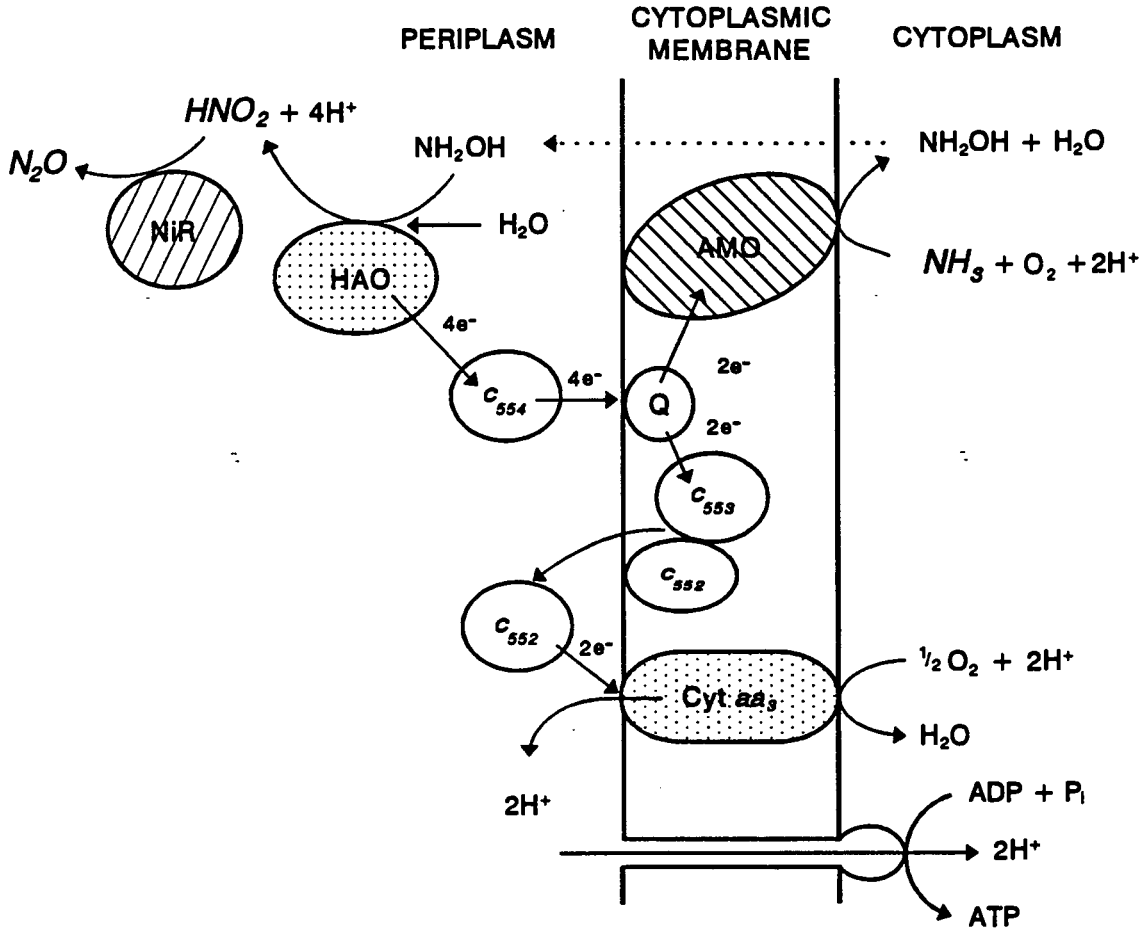

Fig E.1: Arrangement of enzymes of nitrogen oxidation and reduction, and electron transport in membranes of *Nitrosomonas* illustrating that the oxygen molecule incorporated in oxidation of ammonium (NH_3) originates from molecular oxygen (O_2) and the oxygen molecule incorporated in oxidation of hydroxylamine (NH_2OH) originates from water (H_2O). Adapted from Hooper (1989).

[Abbreviations: AMO – ammonium monooxygenase; HAO – hydroxylamine oxidoreductase; NiR – nitrite reductase; Q – Ubiquinone; Cyt – Cytochrome; ADP – adenosine diphosphate; ATP – adenosine triphosphate].

oxidative phosphorylation (Mitchell, 1979). The nett positive charge established by the utilization of protons to form H_2O in the reduction of oxygen (Eq E.5) promotes the flow of protons back to the cytoplasm via a protein called ATPase, which results in the formation of ATP from ADP (see Fig E.1). The mechanism is described fully in Appendix D in dealing with the mechanisms of coupling energy production with denitrification.

(v) Oxygen limitation affects products of ammonia oxidation: It is often noted that products additional to NO_2^- such as nitrous oxide (N_2O) and nitric oxide (NO) are produced in soils and sediments containing nitrifying organisms (Yoshida and Alexander 1970, 1971; Jorgenson *et al.*, 1984). Elkins *et al.* (1978) and Kaplan *et al.* (1978) proposed that increased production of N_2O during nitrification results from low concentrations of dissolved oxygen. On the basis of this proposal Goreau *et al.* (1980) examined the endproducts of the ammonia-oxidizing bacterium *Nitrosomonas* sp. at oxygen concentrations ranging from $\approx$ 7,0 to $\approx$ 0,2 mgO/ℓ. At high concentrations of oxygen, the yield of N_2O was $\approx$ 0,3% that of NO_2^-. At low concentrations of oxygen, the rate of production of NO_2^- decreased 7-fold and the rate of production of N_2O increased 4-fold, such that at concentrations of oxygen of $\approx$ 0,2 mgO/ℓ, the yield of N_2O was nearly 10% that of NO_2^-.

Lipschultz *et al.* (1981) examined the end products of the ammonia-oxidizing bacterium *Nitrosomonas europaea* at oxygen concentrations ranging from $\approx$ 2,0 to $\approx$ 0,2 mgO/ℓ. With decreasing concentration of dissolved oxygen, the endproduct ratio N_2O/NO_2^- increased 2-fold, the ratio NO/NO_2^- increased 7-fold and the ratio NO/N_2O decreased 2-fold, i.e. as the concentration of oxygen decreased, the proportion of NO_2^- decreased, the proportions of NO and N_2O increased and the proportion of N_2O relative to NO increased.

Nitrite reductase: The mechanisms which produce increased proportions of N_2O and NO under conditions of low DO concentration are unclear, but it is apparent that under such conditions, *Nitrosomonas* has a capacity to reduce NO_2^- or NH_2OH to N_2O and NO, in reactions analogous to those found in denitrifying organisms under similar conditions.

From experiments with purified HAO, it became apparent that under conditions of low DO concentration, the first oxidation step (the oxidation of ammonia to hydroxylamine) continues functioning, but the second step (the oxidation of

NO_2^- to NO_3^- and, (iii) the effect of environmental conditions on the oxidation of NO_2^- to NO_3^-.

(i) The source of oxygen for oxidation of nitrite (produced by <u>Nitrosomonas</u>) to nitrate: Conventional stoichiometric reactions indicate molecular oxygen as the oxygen source for this reaction. Aleem *et al.* (1965) using isotopically labelled oxygen atoms in molecular oxygen and water determined that the majority of oxygen atoms in NO_3^- originate from water. The electrons produced during this reaction pass through a series of electron transferring cytochromes and in the final transfer oxygen acts as terminal electron acceptor. To confirm their finding that the oxygen for the oxidation of NO_2^- originates from water and not molecular oxygen, Aleem *et al.* (1965) grew *Nitrobacter* in the absence of oxygen, using ferricyanide as terminal electron acceptor; oxidation of NO_2^- was observed. These results were confirmed by Kumar *et al.* (1983) and Hollocher (1984), with *Nitrobacter agilis* and by Dispirito and Hooper (1986) with *Nitrobacter winogradskyi*, all three groups using isotopic labelling techniques.

The stoichiometric equations describing the oxidation of NO_2^- to NO_3^- and the concomitant reduction of oxygen are as follows:

$$H_2O + HNO_2 \rightarrow HNO_3 + 2H^+ + 2e^- \quad \text{(E.11)}$$

Nitrite dehydrogenase

$$2H^+ + 2e^- + \tfrac{1}{2} O_2 \rightarrow H_2O \quad \text{(E.12)}$$

Cytochrome oxidase

(ii) The enzymes associated with the oxidation of nitrite to nitrate: It was stated above that the electrons originating during the oxidation of NO_2^- pass through a series of electron transferring cytochromes to oxygen. The enzymes associated with the first part of the sequence (the oxidation of NO_2^-) are described as nitrite oxidase, or nitrite dehydrogenase, and have been shown by Faull *et al.* (1969) in work on *Nitrobacter agilis* to have two different activities, a nitrite oxidase ($NO_2^- \rightarrow NO_3^-$) and a nitrate reductase ($NO_3^- \rightarrow NO_2^-$) activity. The results were interpreted as indicative of the existence of two separate enzymes. Similar two-enzyme activities

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[4] Nitrite and nitrate are shown in their associated (HNO_2, and HNO_3) forms and not in their dissociated ($NO_2^- + H^+$) forms as a consequence of the higher affinity of NO_2^- for nitrite dehydrogenase at lower pH (O'Kelly *et al.*, 1970).

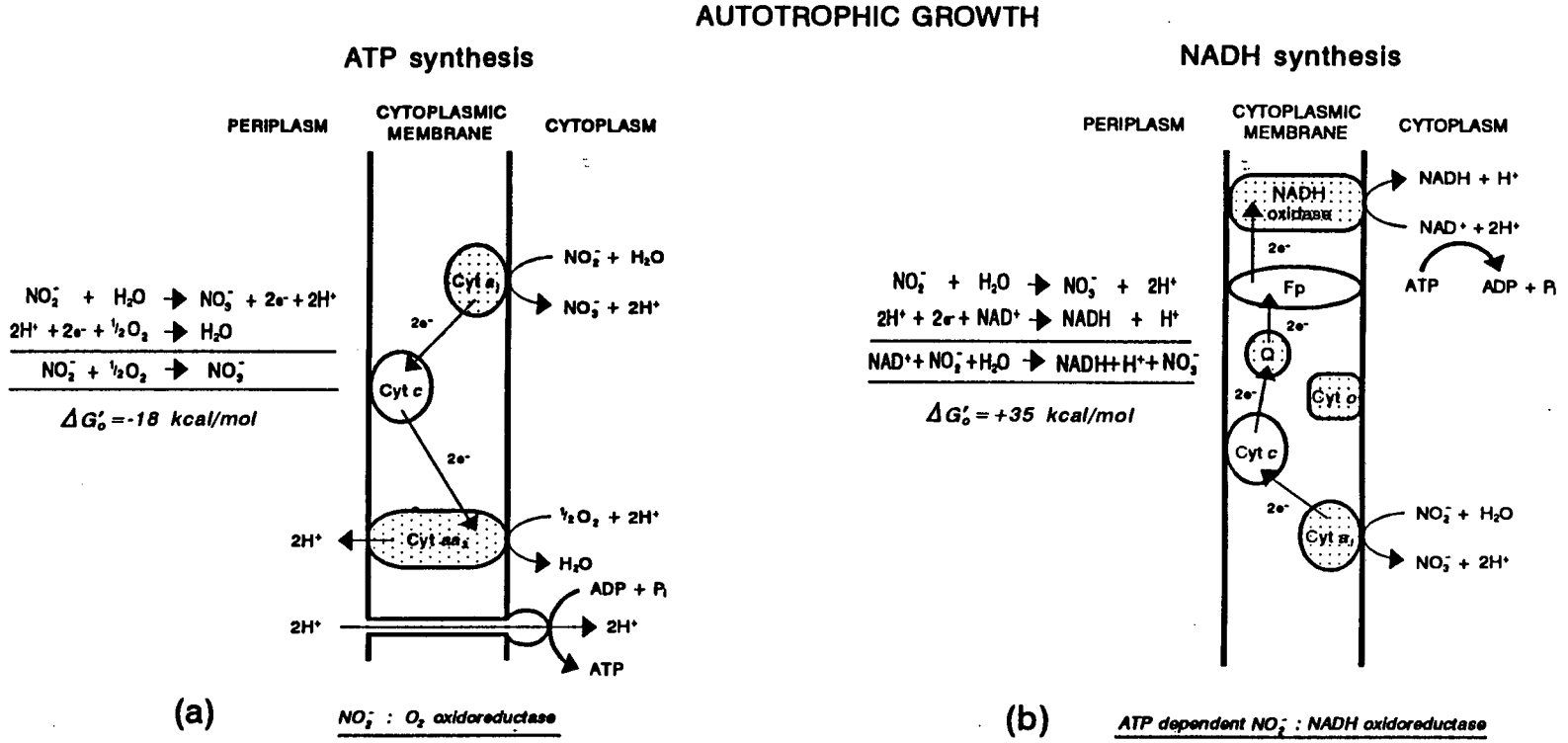

Fig E.2: Hypothesized arrangement of enzymes and electron transport under autotrophic growth conditions, illustrating (a) ATP synthesis through oxidation of nitrite (NO_2^-) to nitrate (NO_3^-) with molecular oxygen as the source of oxygen, and (b) NADH synthesis through reduction of nitrite to nitrate. Adapted from Ferguson (1982), and Aleem and Sewell (1981).

[Abbreviations: ADP – Adenosine diphosphate; ATP – Adenosine triphosphate; Cyt – Cytochrome; F_P – Flavoprotein; NAD^+ – nicotinamide adenine dinucleotide (oxidized); NADH – nicotinamide adenine dinucleotide (reduced); Q – Ubiquinone].

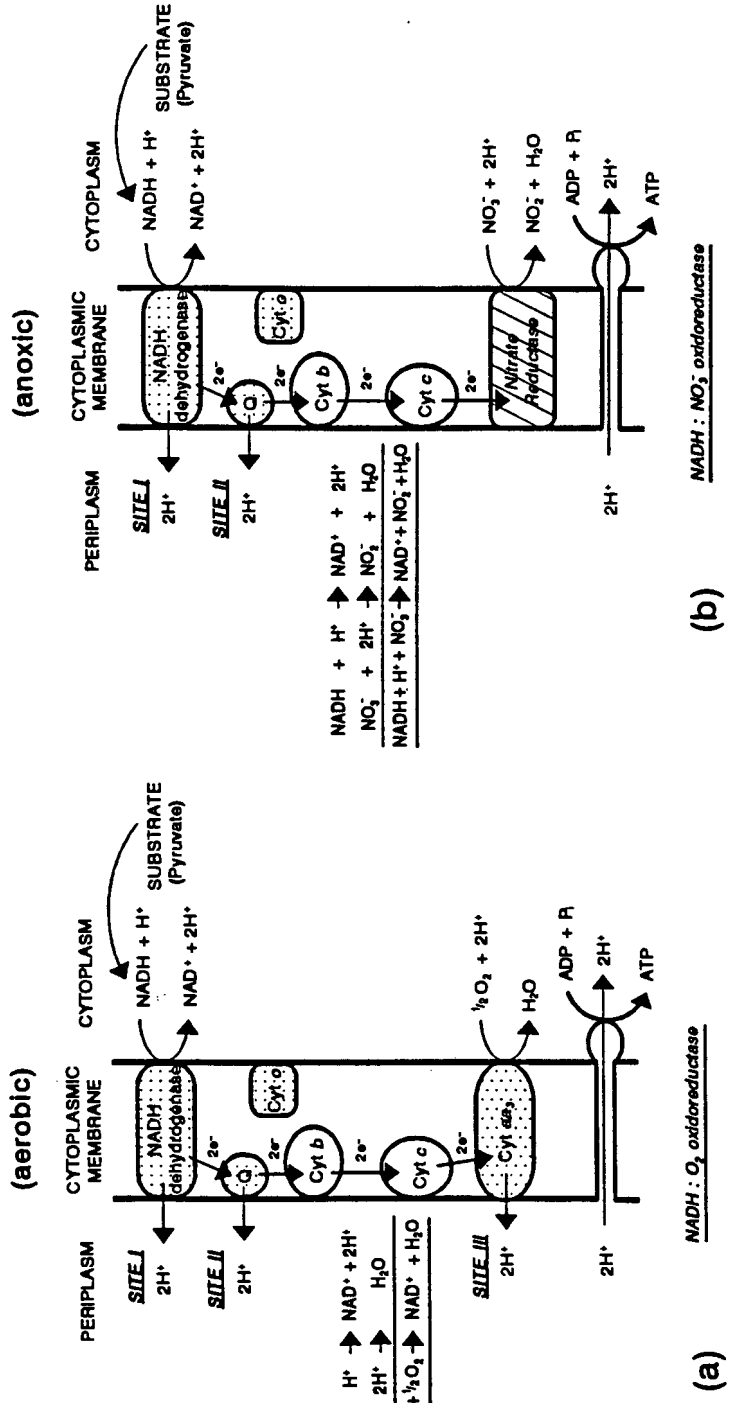

Fig E.3: Hypothesized arrangement of enzymes and electron transport under heterotrophic growth conditions, illustrating (a) ATP synthesis with molecular oxygen (O_2) as electron acceptor (aerobic conditions), and (b) ATP synthesis with nitrate (NO_3^-) as electron acceptor (anoxic conditions). Adapted from Sewell and Aleem (1979) and Aleem and Sewell (1981).

[Abbreviations: ADP – adenosine diphosphate; ATP – adenosine triphosphate; Cyt – Cytochrome; Q – Ubiquinone].

concentration of dissolved oxygen and, (ii) the presence of unaerated periods. Because nitrifiers obtain the majority of their energy under aerobic conditions, their growth is affected by the concentration of dissolved oxygen and the length of time of exposure to anaerobic or anoxic conditions. It is also not unreasonable to suppose that the two nitrifying organisms, *Nitrosomonas* and *Nitrobacter* not only are affected by the concentration of oxygen and the length of time of exposure of the organisms to unaerated conditions but are affected differently by these changes.

(i) The concentration of DO varies in nitrogen and nutrient removal systems either within the reactor (in an intermittently aerated configuration), or between reactors (in an anoxic–aerobic two– or multi–reactor configuration): In a review of the effect of changes in DO on *Nitrosomonas* and *Nitrobacter*, Painter (1970) makes note of the work of Ulken (1963) in which *Nitrobacter* was considered more sensitive to oxygen depletion than *Nitrosomonas*. Schöberl and Engel (1964) found that at 30° C, the growth of *Nitrobacter* was reduced at a DO of 2,0 mgO/ℓ but for *Nitrosomonas*, growth was not affected until the DO fell to 0,9 mgO/ℓ. It is generally accepted that under completely aerobic conditions in which neither NH_3 nor oxygen is limiting and with long residence times, the rate of oxidation by *Nitrosomonas* is frequently the limiting step in nitrification, with the result that the NO_2^- concentration in the medium is essentially nil. However, under conditions in which the dissolved oxygen concentration is below that at which the growth of *Nitrobacter* is affected (DO < 2,0 mgO/ℓ), but above that at which *Nitrosomonas* is affected (DO > 0,9 mgO/ℓ), it is envisaged that the NO_2^- concentration would increase as a result of its formation by *Nitrosomonas* and non-oxidation by *Nitrobacter*.

(ii) In anoxic–aerobic systems, unaerated conditions affect the performance of Nitrosomonas and Nitrobacter under subsequent aerated conditions: McCarty (1964) calculated yield coefficients of 0,29 mgVSS/mg NH_4^+–N for *Nitrosomonas* and 0,084 mgVSS/mgNO_2–N for *Nitrobacter* which equates to proportions of the nitrifier population for *Nitrosomonas* and *Nitrobacter* of 77,5% and 22,5% respectively under steady state aerobic conditions (Gee *et al.*, 1990). Because of the smaller population *of Nitrobacter*, exposure of nitrifier populations to long unaerated periods followed by exposure to aerated periods would lead to the *Nitrosomonas* population reaching steady state numbers under aerobic conditions prior to the *Nitrobacter* population. Thus, at the start of aerobic conditions after anoxic conditions and until steady–state population numbers develop, the disproportion between numbers of

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